

Differences in regulatory T cells between Churg-Strauss syndrome and chronic eosinophilic pneumonia with asthma

Naomi Tsurikisawa, MD, Hiroshi Saito, PhD, Takahiro Tsuburai, MD, Chiyako Oshikata, MD, Emiko Ono, MD, Hiroyuki Mitomi, MD, and Kazuo Akiyama, MD Kanagawa, Japan

Background: Chronic eosinophilic pneumonia (CEP) with asthma precedes the onset of Churg-Strauss syndrome (CSS) in half of all patients with CSS. It is not known what determines whether patients with CEP after asthma will have CSS.

Objective: We examined whether activation of regulatory T cells in patients with CEP inhibits CSS development and is otherwise involved in the mechanism of CSS disease.

Methods: In patients with CSS (n = 38), CEP with asthma (n = 20), and general adult asthma (n = 108), we examined the number of CD4⁺CD25⁺ T cells in peripheral blood, as well as levels of expression of the cytokines IL-2, IL-5, IL-10, and TGF- β by CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells, or both.

Results: At disease onset, patients with CSS, unlike patients with CEP, had significantly fewer CD4⁺CD25⁺ T cells than patients with any step of asthma. CD4⁺CD25⁺ T cells producing IL-10 were rarely detected in patients with CSS at disease onset or relapse, whereas the numbers of IL-10-producing T cells in patients with CEP were high at disease onset. There were fewer CD4⁺CD25⁻ T cells producing IL-2 in patients with CSS before treatment than in patients with CEP at disease onset. The proportions of CD4⁺CD25⁺ T cells producing IL-10 and CD4⁺CD25⁻ T cells producing IL-2 in patients with CSS increased at remission.

Conclusions: Maintenance of the numbers of regulatory T cells in patients with CEP with asthma might inhibit CSS development through the action of cytokines, such as IL-10 and IL-2, produced by CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells. This might be part of a mechanism that influences progression and prognosis in these diseases. (J Allergy Clin Immunol 2008;122:122-128.)

Key words: Churg–Strauss syndrome, regulatory T cells, chronic eosinophilic pneumonia, CD4⁺CD25⁺ T cells, IL-10, TGF- β , IL-2

Churg–Strauss syndrome (CSS) is a rare disease characterized by allergic granulomatosis and necrotizing vasculitis after peripheral blood eosinophilia and eosinophilic tissue infiltration.

Abbreviations used

ACR: American College of Rheumatology
BALF: Bronchoalveolar lavage fluid
CEP: Chronic eosinophilic pneumonia
CSS: Churg–Strauss syndrome
GINA: Global Initiative for Asthma
MNL: Mononuclear leukocyte
nTreg: Naturally occurring regulatory T
PE: Phycoerythrin
Treg: Regulatory T
WBC: Whole blood cell

Asthma is present in 96% to 100% of patients with CSS and is the cardinal feature of CSS. Asthma can precede systemic vasculitis by approximately 8 years and in some cases by more than 30 years.^{1–4} The precise causes of CSS and the asthmatic phase before the onset of CSS are still unknown. However, it is known that eosinophilic infiltrations, such as those found in chronic eosinophilic pneumonia (CEP), eosinophilic gastroenteritis, or both, precede systemic vasculitis in half of all patients with CSS.^{4,5} Some clinicians suggest that patients with CEP after asthma are more likely to have CSS if the CEP is left untreated.⁶ However, some patients in whom asthma is complicated by CEP do not have CSS. There is no clear mechanism that explains why patients with CEP with asthma do or do not have CSS.

The cause of idiopathic CEP is not understood, although in some cases CEP is caused by infection with a parasite, virus, or fungus or by drugs, toxic agents, or radiation therapy.^{7,8} Asthma can occur before CEP in about half to two thirds of patients with CEP.^{7,8} Increasing numbers of eosinophils and lymphocytes in the peripheral blood and bronchoalveolar lavage fluid (BALF) contribute to the development of CEP. There are many mediators of eosinophilia in CEP, including RANTES, eotaxin, thymus and activation-regulated chemokine (CCL17),^{9–11} and cytokines, including IL-2, IL-3, IL-5, IL-10, IL-13, and IL-18.^{11,12} The number of CD4⁺CD25⁺ T cells in BALF and peripheral blood is increased in patients with CEP.¹³ In contrast, PBMCs in patients with CSS secrete not only the type 1 cytokine IFN- γ but also type 2 cytokines, such as IL-4, IL-13, and, in particular, IL-5.¹⁴

Regulatory T (Treg) cells play essential roles in maintaining immunologic homeostasis, preventing autoimmunity, and providing peripheral tolerance against antigens, including autoantigens and allergens. Treg cells, including T_H1 (Treg cell type 1), CD4⁺CD25⁺ cells, T_H3 cells, and natural killer cells, are characterized by their ability to suppress effector T cells of either the T_H1 or T_H2 phenotype involved in mediating inflammation.^{15–17} CD4⁺CD25⁺ cells are naturally occurring regulatory T (nTreg)

From the Clinical Research Center for Allergy and Rheumatology, National Hospital Organization Sagami Hospital, National Hospital.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication November 27, 2007; revised April 3, 2008; accepted for publication May 22, 2008.

Reprint requests: Naomi Tsurikisawa, MD, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization Sagami Hospital, 18-1 Sakuradai, Sagami, Kanagawa, Japan 228-8522. E-mail: n-tsurikisawa@sagami-hosp.gr.jp.

0091-6749/\$34.00

© 2008 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2008.05.040

TABLE I. Patient characteristics

	Asthma				CSS at onset (n = 38)	CEP (n = 20)	P value
	Step 1 (n = 35)	Step 2 (n = 35)	Step 3 (n = 15)	Step 4 (n = 23)			
Age (y), mean $\pm$ SD	52.6 $\pm$ 13.9	52.7 $\pm$ 15.4	45.3 $\pm$ 16.2	53.6 $\pm$ 16.0	56.5 $\pm$ 15.2	57.3 $\pm$ 14.1	NS*
Sex (M/F)	16/19	15/20	9/6	12/11	12/26	8/12	NS†
Age at onset of asthma (y), mean $\pm$ SD	37.1 $\pm$ 21.0	41.9 $\pm$ 19.8	27.4 $\pm$ 18.2	38.8 $\pm$ 16.6	43.5 $\pm$ 15.7	46.9 $\pm$ 14.0	NS*
Age at onset of CSS (y), mean $\pm$ SD					53.8 $\pm$ 16.5		
Age at onset of CEP (y), mean $\pm$ SD						53.6 $\pm$ 15.4	
Type: atopic/nonatopic	18/17	19/16	8/7	13/10	11/27	7/13	NS†
Step 1/2/3/4 at the first hospital visit					2/3/1/32	3/0/2/15	NS†
Daily dose of ICS (μ g), converted to FP equivalents	0	182.9 $\pm$ 38.3	400 $\pm$ 0	841.7 $\pm$ 166.6‡	619.1 $\pm$ 418.9‡	730.0 $\pm$ 426.9‡	.01‡
Daily dose of OCS (mg), converted to prednisolone equivalents	0	0	0	5.0 (2 patients)	8.0 (5 patients)	5.0 (2 patients)	

NS, Not significant; ICS, inhaled corticosteroids; FP, fluticasone propionate; OCS, oral corticosteroids.

*Two-way ANOVA with repeated measures among all groups.

† χ^2 Testing revealed no significant differences between the frequencies in each group.

‡Step 4 asthma, CSS, or CEP versus steps 1, 2, and 3: $P < .01$.

cells first characterized by Sakaguchi et al¹⁸ in 1995; since then, human nTreg cells have been defined as CD25^{hi}CD127^{low}FOXP3⁺.¹⁹ nTreg cells can mediate their suppressive effects through cell contact.²⁰ T_R1 cells are antigen-specific populations derived from conventional CD4⁺CD25[−] naive precursors after exposure to antigen under conditions of limiting costimulation, such as with IL-10, immature dendritic cells, or dexamethasone; the mechanism by which T_R1 cells exert suppression appears to be mediated by secretion of suppressor cytokines, such as IL-10 and TGF- β .

Recent studies have focused on Treg cells and the mechanisms of human diseases, such as systemic lupus erythematosus,^{21,22} rheumatoid arthritis,^{23,24} cancer,^{25,26} Guillain-Barré syndrome,²⁷ multiple sclerosis,²⁸ infection,^{29,30} and asthma,³¹⁻³⁵ but it is not known whether Treg cell activity is related to the mechanism and cause of CSS.

We examined the phenotypes and functions of CD4⁺CD25⁺ T cells in CEP with asthma and in CSS at onset and after therapy, and we compared these observations with those in general asthma. Activation of Treg cells in patients with CEP was investigated to determine whether these cells can inhibit the development of CSS and play a role in the mechanism of the disease.

METHODS

Patients

We recruited 38 patients with CSS, 20 patients with CEP with asthma, and 108 adult patients with general asthma (non-CSS and non-CEP) treated between January 2003 and May 2007 at the Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagami-hara, Kanagawa, Japan. All 108 adult patients with general asthma were given diagnoses according to the criteria of the Global Initiative for Asthma (GINA) guidelines,³⁶ and the severity of their asthma was classified as follows, as defined by GINA guidelines: step 1, intermittent; step 2, mild persistent; step 3, moderate persistent; and step 4, severe persistent. Criteria for CSS were defined according to the classification of the American College of

Rheumatology (ACR).³⁷ The ACR uses 6 criteria: asthma, eosinophilia, polyneuropathy, pulmonary infiltrates, paranasal sinus abnormality, and the presence of extravascular eosinophils. The ACR definition of CSS is the presence of 4 of the 6 criteria. Patients were classified into a relapse group and a remission group in accordance with the condition of the disease after treatment. The remission state was defined as the absence of any clinical signs or symptoms of active vasculitis. The relapse state was defined as the recurrence of vasculitis symptoms with or without an increase in the proportion of eosinophils among whole blood cells (WBCs) after initial remission requiring the resumption of immunosuppressive therapy or an increased dose of immunosuppressant. In all patients with CEP, the CEP was complicated by asthma. There were 3 diagnostic criteria for CEP: respiratory symptoms of more than 2 weeks' duration; alveolar or blood eosinophilia (alveolar eosinophilia >25% on BALF differential cell count and blood eosinophilia >1000/mm³); and pulmonary infiltrates with a usually peripheral predominance on chest imaging.⁷ Patients with CEP who had allergic bronchopulmonary mycosis were excluded from the study.

In all patients with asthma, CSS, or CEP, as well as in healthy control subjects (n = 9; age range, 27-52 years), we examined the numbers of eosinophils and WBCs in the peripheral blood and the proportions of CD4⁺CD25⁺ T cells. We also examined expression of the cytokines IL-2, IL-5, IL-10, and TGF- β by CD4⁺CD25⁺ T cells at the time of disease onset and in the relapse and remission groups in patients with CSS, at the time of disease onset and after disease improvement in patients with CEP, and at the time without disease exacerbation in asthmatic patients. Only 5 of 38 patients with CSS had already received oral corticosteroids (mean, 8.0 mg prednisolone equivalent; Table I) and other antiasthma drugs, such as inhaled corticosteroids, long-acting β -stimulants, leukotriene antagonists, and theophylline. However, the remaining 33 of the 38 patients had received treatment with other antiasthma drugs, except oral corticosteroids. The study was approved by the ethics committee of our hospital, and informed consent was obtained from each patient.

Immunologic analysis

Reagents. Fluorescein isothiocyanate-conjugated mouse IgG1, anti-CD4, and anti-CD25; phycoerythrin (PE)-conjugated mouse IgG1, anti-CD4, anti-CD25; and PE-Cy5-conjugated mouse IgG1, and anti-CD4 antibody were purchased from BioLegend (San Diego, Calif). PE-conjugated anti-human

IL-2, IL-5, and IL-10 were purchased from BD Biosciences (Nihon BD, Tokyo, Japan), and PE-anti-human TGF- β was purchased from IQ-Products (Groningen, The Netherlands). Peridinin-chlorophyll-protein (PerCP)-conjugated mouse IgG1 and anti-CD4 antibodies were purchased from BD Biosciences.

Ratio of CD25 positivity in CD4⁺ T-cell analysis. The percentage of CD4⁺ T cells was counted in the peripheral blood, and the percentage expression of CD25⁺ molecules was examined in CD4⁺ T cells. By using the method of Landay and Muirhead,³⁸ mononuclear leukocytes (MNLs) were incubated with fluorescein isothiocyanate-conjugated anti-CD4 and PE-conjugated anti-CD25. The surface markers of the CD4⁺ T cells were analyzed by means of flow cytometry (FACSCalibur; Nippon Becton Dickinson, Tokyo, Japan).

Intracellular cytokine staining: Cytokine production and FACS analysis. MNLs obtained from the patients were stimulated for 4 hours with medium or 20 ng/mL phorbol 12-myristate 13-acetate and 1 μ g/mL ionomycin at 37°C in the presence of 10 μ g/mL brefeldin A to generate and accumulate cytokines.³⁹ The surface-stained MNLs were suspended in 0.5 mL of cold 4% paraformaldehyde fixative and incubated at room temperature for 10 minutes. After fixation, cells were washed twice with PBS and spun at 200g for 7 minutes. The cell pellet was harvested and resuspended in 2 mL of serum amyloid P (0.1% [wt/vol] saponin and 0.05% [wt/vol] NaN₃ in Hanks's balanced salt solution) buffer. The cells were centrifuged at 200g for 7 minutes, and then the pellet was resuspended in 0.1 mL of serum amyloid P buffer. PE-conjugated anti-human cytokine antibodies (IL-2, IL-5, IL-10, and TGF- β) were added (10⁶ cells in 20 μ L) to each tube. The tube was vortexed and then incubated for 35 minutes at room temperature in the dark. The proportion of cells generating cytokines was measured by using the FACSCalibur and was analyzed with CELLQuest software (Nippon Becton Dickinson). T_H1 cells were defined as CD4⁺CD25⁺ T cells predominantly producing IL-10 and TGF- β .¹⁵

Statistical analysis

All values are expressed as means $\pm$ SDs unless otherwise specified. Statistical comparisons between groups were carried out by means of 2-way ANOVA with repeated measures, followed by a *post hoc* comparison by using the Newman-Keuls test. Two mean values were compared by using the Mann-Whitney *U* test. *P* values of less than .05 were considered statistically significant. Statistical analysis was performed with the StatView 5.0 statistical program (SAS Institute, Inc, Cary, NC).

RESULTS

Clinical findings

There were more women than men with CSS and CEP, but there was no significant difference in the sex ratio among patients with asthma at any step. Thirty-eight patients with CSS and 15 of the 20 patients with CEP had been classified according to the GINA guidelines as having severe asthma at the first hospital visit (Table I). There was no significant difference in the age of onset of asthma between those who had CSS or CEP. Patients with CEP and asthma did not go on to have CSS during the course of the study. Twelve patients with CEP showed improved pulmonary infiltration and decreased eosinophil counts as a proportion of total WBC counts in blood after treatment with systemic corticosteroids, and these parameters improved in a further 8 patients with CEP after continuation of asthma therapy without systemic corticosteroids. After improvement of eosinophilic pneumonia, the eosinophil count in the peripheral blood decreased from a mean of 3762.6 cells/L to a mean of 440.1 cells/L (Table II). Eleven of 38 patients with CSS had positive results for myeloperoxidase-specific antineutrophil cytoplasmic autoantibody. Four of 24 patients with CSS had positive results for immune complex (>1.5 μ g). The total WBC and eosinophil

TABLE II. Hematologic, BALF, and sputum cell counts in patients with CEP and asthma (n = 20)

Differential cell counts in BALF, median (range)	
Total cells ($\times 10^6$ /mL)	3.3 (0.2–5.6)
Macrophages (%)	45.8 (18–76.7)
Lymphocytes (%)	27.6 (5.4–43.0)
Neutrophils (%)	1.7 (0–4.0)
Eosinophils (%)	24.3 (7–39.0)
Mast cells (%)	1.1 (0–6)
Eosinophils in sputum by Hansel's classification,* no. of patients (%)	
Mild	1 (6.7)
Moderate	5 (33.3)
Severe	9 (60.0)
WBC ($\times 10^3$ cells/L), median (range)	
At disease onset	10,028.0† (4340–16,800)
After improvement	7531.8 (4400–11,180)
Eosinophils in blood (cells/L), median (range)	
At disease onset	3762.6‡ (1124–8113)
After improvement	440.1 (50–640)

*According to the Hansel classification, the level of eosinophilia was scored as mild, no, or few eosinophils throughout the entire smear; moderate, which was defined as several eosinophils in each high-power ($\times 400$) microscopic field; and severe, which was defined as numerous eosinophils in each high-power microscopic field.

†At disease onset versus after improvement: *P* < .05.

‡At disease onset versus after improvement: *P* < .01.

counts were significantly reduced at the time of remission or relapse compared with those at the time of onset, but they did not differ significantly between remission and relapse in patients with CSS. The number of organs involved that presented with vasculitis (including active symptoms of asthma or sinusitis) was significantly greater in patients with CSS at relapse (2.9 ± 0.9) than in those in remission (1.2 ± 0.7 , *P* < .01; Table III). The mean eosinophil count in the peripheral blood in patients at CSS onset was 8042.6 cells/L, which is much greater than that in patients with CEP (Table IV).

Percentage of CD25 positivity in CD4⁺ T cells

The percentage of CD25 positivity in CD4⁺ T cells (CD4⁺CD25⁺) was not significantly different among steps 1, 2, and 3 of asthma, but positivity in step 4 of asthma was significantly lower than in steps 1 and 2 (*P* < .01). Patients with CSS at onset had significantly lower levels of CD4⁺CD25⁺ T cells than asthmatic subjects at any step or patients with CEP at onset. In contrast, the CD4⁺CD25⁺ T-cell count in patients with CEP at onset was the same as in asthmatic subjects at every step of asthma (Table IV) and did not change after improvement of the disease. The CD4⁺CD25⁺ T-cell count in patients with CSS increased after treatment for the disease, and this increase was significantly greater in patients in remission than in those with relapse (*P* < 0.01, Fig 1).

Intracellular cytokine staining: Cytokine production and FACS analysis

The percentage of CD4⁺CD25⁺ T cells producing IL-5 was high in patients with CSS and CEP at onset, significantly greater than at any step of asthma. The percentages of CD4⁺CD25⁺ and CD4⁺CD25[−] T cells producing IL-10 were significantly greater in patients with CEP at onset than in those with CSS at onset or

TABLE III. Hematologic and disease characteristics of patients with CSS (n = 38)

WBCs ($\times 10^3$ cells/L), median (range)	
At onset	15,821.4* (6740-31,800)
At relapse	7909.7 (3300-15,780)
At remission	6277.5 (3220-11,590)
Eosinophil count in blood (cells/L), median (range)	
At onset	8042.6* (370-25,122)
At relapse	367.2 (30-3310)
At remission	182.9 (20-860)
MPO-ANCA (%) at onset	28.9
PR3-ANCA (%) at onset	0
IC (%) >1.5 mg/mL	16.7
Organ involvement at disease onset (%)	
Asthma	100
Paranasal sinusitis	89.4
Multiple polyneuropathy	100
Pulmonary infiltrates	75
Myocardial involvement	73.7
Gastrointestinal tract	81.6
Renal involvement	36.8
Skin involvement	59.3
Central nervous system involvement	21.1
Extravascular eosinophilia (pathologic)	76.3
ACR 4/6(%)	7.9
ACR 5/6(%)	26.3
ACR 6/6(%)	65.8
Cumulative organ involvement (including asthma and sinusitis), no. $\pm$ SD	
At onset	7.4 $\pm$ 1.6*
At relapse	2.9 $\pm$ 0.8†
At remission	1.2 $\pm$ 0.7

MPO-ANCA, Myeloperoxidase-specific antineutrophil cytoplasmic autoantibody; PR3-ANCA, proteinase-3 antineutrophil cytoplasmic autoantibody; IC, immune complex; ACR, American College of Rheumatology criteria (see text).

*At onset versus at relapse and at remission: $P < .01$.

†At relapse versus at remission: $P < .01$.

in asthmatic subjects at any step ($P < .01$). The percentage of CD4⁺CD25⁺ T cells producing TGF- β was significantly greater in patients with CSS at onset than in steps 1 or 2 of asthma. However, TGF- β production was significantly greater in patients with CEP at onset than in those with CSS at onset ($P < .01$). The percentage of CD4⁺CD25⁻ T cells producing IL-2 was significantly lower in patients with step 4 asthma than in those with step 1 ($P < .01$) or step 2 ($P < .05$) asthma. The percentage of CD4⁺CD25⁻ T cells producing IL-2 was significantly lower in patients with CSS at onset than in those with asthma at steps 1 or 2 or with CEP at onset ($P < 0.01$, Table IV). The percentage of CD25 positivity in CD4⁺ T cells was correlated with IL-10 production in all patients with stable asthma or with CSS or CEP at onset ($R = 0.42$, $P < .01$; data not shown). These results showed that the CD4⁺CD25⁺ T cells producing IL-10 were T_R1 cells.

Moreover, we examined the levels of the cytokines IL-10 and TGF- β in serum. The levels of IL-10 in serum were as follows: CSS at onset versus CSS in remission, 1.7 ± 1.1 versus 1.1 ± 1.2 pg/mL (not significant); CEP at onset versus CEP after improvement, 1.0 ± 1.6 versus 0.5 ± 0.7 pg/mL (not significant). Levels of TGF- β in the serum were as follows: CSS at onset versus CSS in remission, 7.2 ± 4.3 versus 7.5 ± 7.3 ng/mL (not significant); CEP at onset versus CEP after improvement, 14.6 ± 11.2 versus 12.1 ± 10.6 ng/mL (not significant). Serum levels of IL-10 were

extremely low. We cannot work out how there were no significant differences among the serum levels of these cytokines, but there were significant differences among the intracellular levels.

The count of CD4⁺CD25⁺ T cells producing IL-5, which causes eosinophilia in peripheral blood cells and lung tissue, was high in patients with CSS and CEP at disease onset and decreased significantly after treatment of CSS and improvement of CEP (Fig 2, A). CD4⁺CD25⁺ T cells producing IL-10 were rarely detected in patients with CSS at onset or in the relapse state. However, when patients with CSS had clinical remission after treatment, the number of CD4⁺CD25⁺ T cells producing IL-10 significantly increased compared with those at onset. The count of CD4⁺CD25⁺ T cells producing IL-10 in patients with CEP was high at disease onset and then decreased significantly after improvement of eosinophilic pneumonia (Fig 2, B). The count of CD4⁺CD25⁻ T cells producing IL-2 in patients with CSS at onset was fewer than in patients with CEP at onset. After treatment of CSS, patients who reached remission showed an increase in the number of CD4⁺CD25⁻ T cells producing IL-2 to the level seen in patients with CEP at disease onset, but there was no change in patients with CEP between disease onset and improvement (Fig 2, C). The number and distribution of organs involved was significantly negatively correlated with the percentage of CD25 positivity of CD4⁺ T cells at onset, remission, and relapse in patients with CSS ($r = -0.51$, $P < .01$; Fig 3).

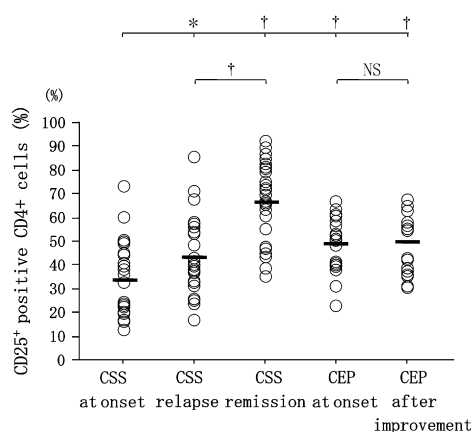
DISCUSSION

CSS is a rare vasculitis with an annual incidence of approximately 2.4 to 6.8 cases per 1 million persons.^{40,41} The report by Guillevin et al² revealed some triggering factors that preceded the onset of the disease by up to several months, including desensitization, rapid discontinuation of oral corticosteroids, and vaccination. The cause and pathogenesis, and especially the heritability, of CSS are still unknown. In a rare case of familial CSS in 2 sisters, it was not possible to explain the heritability of the disease from HLA typing.⁴² Moreover, the characteristics of the asthma preceding the onset of CSS are still unknown. At the initial hospital visit for asthma symptoms before the onset of CSS, patients were likely to be adult, of female sex, and nonatopic and with asthma of severe grade and eosinophils present at greater than 20% of WBCs.⁴³ The FEV₁ in patients who had CSS was as low as that seen in patients with severe asthma, but bronchial hyperresponsiveness to acetylcholine in patients who had CSS was as slight as in those with mild asthma.⁴³ These results suggest that the asthma in patients who might go on to have CSS is different from that seen in general asthmatic subjects. Most patients before CEP onset are also severely asthmatic, but the difference between their asthma and that seen before CSS is unknown.

CSS is an eosinophilic systemic vasculitis characterized by peripheral blood eosinophilia. Eosinophils are considered to be the most important effector cells and contribute to the mechanism in this disease.^{1,2,6-8} CD69 and CD25 are surface markers of eosinophil activation that are expressed on eosinophils, and there is an increase in CD69 expression on eosinophils in the peripheral blood of patients with CSS.⁴⁴ There are many mediators related to increased eosinophil count, including IL-3, IL-5, GM-CSF, RANTES, and eotaxin. The numbers of CD4⁺CD25⁺ T cells producing IL-5 were high in patients with CSS and CEP at disease onset; they decreased after treatment of CSS and improvement in CEP. However, it has been reported that an increase in the

TABLE IV. Blood eosinophil count and cytokines (percentage of expression) in CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells

	Healthy control subjects (n = 9)	BA				CSS at onset	CEP at onset
		Step 1	Step 2	Step 3	Step 4		
Blood eosinophils (no. of cells)	161.9 ± 65.0	209.4 ± 168.3	290.6 ± 209.8	357.3 ± 215.5	380.0 ± 517.0	8042.6 ± 6559.3*†	3762.6 ± 2310.3*
CD4 ⁺ CD25 ⁺ cells (%)	31.4 ± 8.2‡	52.2 ± 12.2	52.6 ± 11.8	46.2 ± 6.6	42.7 ± 10.3§	34.9 ± 15.6*†	48.1 ± 11.6
IL-5 in CD4 ⁺ CD25 ⁺ cells (%)	0.0 ± 0.0	4.4 ± 6.6	5.2 ± 10.1	6.2 ± 10.8	4.1 ± 7.4	14.6 ± 11.9	11.2 ± 12.5¶
IL-10 in CD4 ⁺ CD25 ⁺ cells (%)	0.0 ± 0.0	4.7 ± 7.5	4.9 ± 6.3	1.1 ± 2.2	2.7 ± 4.4	0.3 ± 1.0#	20.9 ± 17.0*†
IL-10 in CD4 ⁺ CD25 ⁻ cells (%)	0.5 ± 1.2	0.5 ± 1.5	0.4 ± 0.6	0.2 ± 0.5	0.3 ± 0.5	0.1 ± 0.1	6.6 ± 11.4*†
TGF-β in CD4 ⁺ CD25 ⁺ cells (%)	0.0 ± 0.0**	6.7 ± 10.0	4.4 ± 6.9	2.8 ± 5.0	2.6 ± 5.9	1.0 ± 0.9††	6.7 ± 10.5†
IL-2 in CD4 ⁺ CD25 ⁻ cells (%)	26.3 ± 25.8	35.1 ± 17.9	32.4 ± 22.3	23.1 ± 21.2	17.0 ± 16.5‡‡	7.6 ± 7.2††	22.0 ± 17.0†

*CSS or CEP versus control and steps 1, 2, 3, and 4: $P < .01$.†CSS versus CEP: $P < .01$.‡Control versus steps 1, 2, and 3 and CEP: $P < .01$; versus step 4: $P < .05$.§Step 4 versus steps 1 and 2: $P < .01$.||CSS versus control and steps 1, 2, and 4: $P < .01$; versus step 3: $P < .05$.¶CEP versus control: $P < .01$; versus steps 1, 2, and 4: $P < .05$.#CSS versus steps 1 and 2: $P < .05$.**Control versus steps 1, 2, 3, and 4 and CEP: $P < .01$.††CSS versus steps 1 and 2: $P < .01$.‡‡Step 4 versus step 1: $P < .01$; versus step 2: $P < .05$.**FIG 1.** Percentage of CD25 positivity in CD4⁺ T cells in patients with CSS and CEP, respectively, at disease onset and after treatment. Median values are shown by horizontal bars. Differences between patient groups were evaluated by using the Mann-Whitney *U* test. † $P < .01$ and * $P < .05$. NS, Not significant.

eosinophil count in the peripheral blood is not related to signs of relapse of CSS.⁴⁵ Moreover, the number of CD4⁺CD25⁺ T cells producing IL-5 did not increase during the relapse phase of CSS. This suggests that the essential mechanisms of changes in disease activity, such as relapse or remission, are related to the regulation of T cells more than to eosinophil activity.

The number of CD4⁺CD25⁺ T cells in patients with CSS at disease onset was lower than in any of the steps of asthma or in CEP at onset. It increased after treatment of the disease, and it was significantly higher in patients in remission than in those who relapsed. In contrast, the number of CD4⁺CD25⁺ T cells in patients with CEP at disease onset did not change after disease improvement. We examined the percentage of FOXP3 that

indicated nTreg cells staining in CD4⁺CD25⁺ T cells. It did not differ significantly among healthy control subjects versus CEP at onset versus CEP after improvement: 4.7% ± 2.6% versus 4.8% ± 1.7% versus 3.7% ± 2.4%, respectively. This lack of change in CD4⁺CD25⁺ T cells after improvement in CEP was due to the production and supply of IL-2 by the CD4⁺CD25⁻ T cells, an event that induced the generation and maintenance of Treg cells. There is an increase in the number of CD4⁺CD25⁺ T cells in asthmatic subjects during acute exacerbations,³¹ whereas the number of CD4⁺CD25^{hi} T cells in the BALF and peripheral blood of children with asthma is lower than in control subjects.^{34,35} Although the numbers of CD4⁺CD25⁺ T cells in patients with CSS at disease onset were significantly lower than in every step of asthma, numbers of these cells in patients with CEP did not decrease and continue at high levels similar to those found in general asthma. In addition, the number of CD4⁺CD25⁺ T cells producing IL-10 in patients with CEP was significantly higher at disease onset than in CSS at disease onset and in a relapse state. Therefore it is possible that CD4⁺CD25⁺ T cells are mainly T_H1 cells and make up part of the nTreg cell population.

Astier et al⁴⁶ indicated that CD4⁺CD25^{hi} cells stimulated by CD46 molecules in patients with multiple sclerosis could not secrete IL-10, although stimulation by CD28 molecules could produce IL-10 from CD4⁺CD25^{hi} cells. We confirmed, using the method of Astier et al, that IL-10 could be produced by CD4⁺CD25^{hi} T cells in 3 patients with CSS in relapse. The percentages of positive CD4⁺CD25⁺ cells that produced IL-10 were 2.3% ± 4.0% for incubation with anti-mouse IgG1 plus IL-2, 6.6% ± 5.8% for incubation with anti-CD3 antibody plus IL-2, 17.2% ± 11.7% for incubation with anti-CD3 antibody plus IL-2 plus anti-CD28 antibody, and 24.7% ± 9.8% for incubation with anti-CD3 antibody plus IL-2 plus anti-CD46 antibody. Interestingly, these results indicated that CD4⁺CD25⁺ T cells could produce IL-10 in patients with CSS in relapse in response to

The percentage of CD25⁺ or CD25⁻ in the CD4⁺ T cells which produced these cytokines

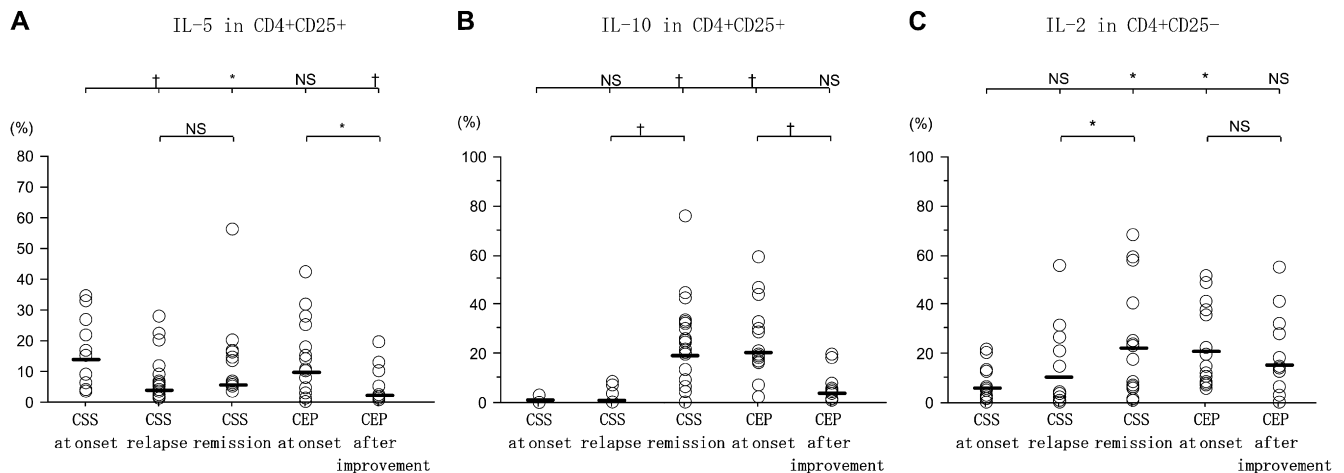


FIG 2. Percentages of CD4⁺CD25⁺ T cells producing IL-5 (**A**), CD4⁺CD25⁺ T cells producing IL-10 (**B**), and CD4⁺CD25⁻ T cells producing IL-2 (**C**) in patients with CSS or CEP at disease onset and after treatment. Median values are shown by horizontal bars. Differences between patient groups were evaluated by using the Mann-Whitney *U* test. †*P* < .01 and **P* < .05. NS, Not significant.

The correlation of CD25⁺ in the CD4⁺ T cells and the number of distribution of organ involvements

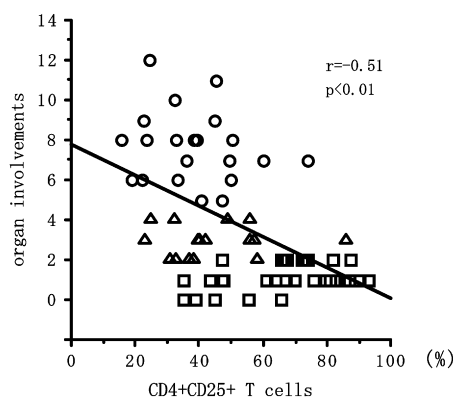


FIG 3. Correlation between the percentage of CD25 positivity of CD4⁺ T cells and the number and distribution of involved organs. Organ involvement included asthma, sinusitis, pulmonary infiltration, mononeuritis multiplex or polyneuropathy, myocardial involvement, gastrointestinal tract involvement, renal involvement, skin involvement, central nervous system involvement, arthritis, tympanitis, eye involvement, gallbladder involvement, liver involvement, and lymphadenitis, mastitis. Open circles represent patients with CSS at onset, open triangles represent patients with CSS at relapse after initial remission, and open squares represent patients with CSS at remission after treatment. A significant linear relationship was evident ($r = -0.51$, $P < .01$) for all patient groups.

stimulation not only with CD28 but also with CD46. This IL-10 production by CD4⁺CD25⁺ T cells and other mechanisms differentiates the T_R1 cells in CSS from those in multiple sclerosis.

Eight of 20 patients with CEP improved after continuing asthma therapy without systemic corticosteroids. The percentages of CD4⁺CD25⁺ T cells producing IL-5 and IL-10 in these patients decreased after improvement in the pulmonary infiltration and eosinophilia in the peripheral blood. These results suggest that Treg cells in patients with CEP inhibit the development of CSS and that the suppressive function of Treg cells in CEP acted

naturally in the absence of systemic corticosteroids. Thus a decrease in the number of CD4⁺CD25⁺ T cells and in the amounts of IL-10 and TGF- β produced by these cells regulated the disease condition and cause of CSS by suppression of Treg activity.

IL-2 is indispensable for the peripheral maintenance of CD4⁺CD25⁺ nTreg cells. The principal physiologic source of IL-2 for maintenance of Treg cells appears to be CD25^{lo}CD4⁺ activated T cells.⁴⁷ In addition, IL-2 is essential for TGF- β to convert naive CD4⁺CD25⁻ T cells to T_R1 cells and for the expansion of these cells.⁴⁸ Our results showed that numbers of CD4⁺CD25⁻ T cells producing IL-2 were lower in CSS at onset than in step 1 and 2 asthma or CEP at onset. Numbers of CD4⁺CD25⁻ T cells producing IL-2 in CEP at onset did not decrease but remained similar to those in every step of asthma. IL-2 provided by CD4⁺CD25⁻ T cells maintains the survival of nTreg cells and induces T_R1 cell production through the upregulation of CD25⁺ and production of IL-10. This indicates that T_R1 cells in patients with CEP might maintain suppressive function from the onset of disease, and therefore their activity might be able to inhibit the development of CSS. Numbers of CD4⁺CD25⁺ T cells producing IL-10 and CD4⁺CD25⁻ T cells producing IL-2 in our patients with CSS increased after treatment with corticosteroids and immunosuppressive agents.

The lack of decrease in the number of Treg cells in CEP might thus inhibit the development of CSS through the production of cytokines, such as IL-10, TGF- β , and IL-2, and these events might contribute to the development of CSS and the mechanisms of the diseases. In the future, these clinical studies and immunologic studies of these issues should elucidate the mechanisms of onset of CSS and its prognosis.

Key message

- Maintenance of the numbers of Treg cells in patients with CEP with asthma might inhibit CSS development through the action of cytokines, such as IL-10 and IL-2, produced by CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells.

REFERENCES

- Churg J, Strauss L. Allergic granulomatosis, allergic angiitis, and periarteritis nodosa. *Am J Pathol* 1951;27:277-301.
- Guillemin L, Cohen P, Casassus P, Lhote F, Jarrousse B. Churg-Strauss syndrome. Clinical study and long-term follow-up of 96 patients. *Medicine* 1999;78:26-37.
- Chumbley L, Harrison E, DeRemee R. Allergic granulomatosis and angiitis (Churg-Strauss syndrome). *Mayo Clin Proc* 1977;52:477-84.
- Lhote F, Guillemin L. Polyarteritis nodosa, microscopic polyangiitis, and Churg-Strauss syndrome. Clinical aspects and treatment. *Rheum Dis Clin North Am* 1995;21:911-47.
- Steinfeld S, Golstein M, De Vuyst P. Chronic eosinophilic pneumonia (CEP) as a presenting feature of Churg-Strauss syndrome (CSS). *Eur Respir J* 1994;7:2098.
- Churg A. Recent advances in the diagnosis of Churg-Strauss syndrome. *Mod Pathol* 2001;14:1284-93.
- Marchand E, Cordier JF. Idiopathic chronic eosinophilic pneumonia. *Semin Respir Crit Care Med* 2006;27:134-41.
- Cottin V, Cordier JF. Eosinophilic pneumonias. *Allergy* 2005;60:841-57.
- Kurashima K, Mukaida N, Fujimura M, Yasui M, Shinagawa T, Matsuda T, et al. A specific elevation of RANTES in bronchoalveolar lavage fluids of patients with chronic eosinophilic pneumonia. *Lab Invest* 1997;76:67-75.
- Katoh S, Matsumoto N, Fukushima K, Mukae H, Kadota JI, Kohno S, et al. Elevated chemokine levels in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia. *J Allergy Clin Immunol* 2000;106:730-6.
- Miyazaki E, Nureki S, Fukami T, Shigenaga T, Ando M, Ito K, et al. Elevated levels of thymus- and activation- regulated chemokine in bronchoalveolar lavage fluid from patients with eosinophilic pneumonia. *Am J Respir Crit Care Med* 2002;165:1125-31.
- Katoh S, Matsumoto N, Matsumoto K, Fukushima K, Matsukura S. Elevated interleukin-18 levels in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia. *Allergy* 2004;59:850-6.
- Albera C, Ghio P, Solidoro P, Mabritto I, Marchetti L, Pozzi E. Activated and memory alveolar T-lymphocytes in idiopathic eosinophilic pneumonia. *Eur Respir J* 1995;8:1281-5.
- Kiene M, Csernok E, Mueller A, Metzler C, Trabandt A, Gross WL. Elevated interleukin-4 and interleukin-13 production by T cell lines from patients with Churg-Strauss syndrome. *Arthritis Rheum* 2001;44:469-73.
- Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000;101:455-8.
- Jonuleit H, Schmitt E, Kakirman H, Stassen M, Knop J, Enk AH. Infectious tolerance: human CD25(+) regulatory T cells convey suppressor activity to conventional CD4(+) T helper cells. *J Exp Med* 2002;196:255-60.
- Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* 2001;182:207-14.
- 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.
- Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol* 2007;7:585-98.
- Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629-44.
- Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 2007;178:2579-88.
- Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, et al. Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2006;27:110-8.
- Behrens F, Himsel A, Rehart S, Stanczyk J, Beutel B, Zimmermann SY, et al. Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis. *Ann Rheum Dis* 2007;66:1151-6.
- van Amelsfort JM, van Roon JA, Noordegraaf M, Jacobs KM, Bijlsma JW, Lafeber FP, et al. Proinflammatory mediator-induced reversal of CD4+CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis Rheum* 2007;56:732-42.
- Nummer D, Suri-Payer E, Schmitz-Winnenthal H, Bonertz A, Galindo L, Antolovich D, et al. Role of tumor endothelium in CD4+CD25+ regulatory T cell infiltration of human pancreatic carcinoma. *J Natl Cancer Inst* 2007;99:1188-99.
- Li H, Yu JP, Cao S, Wei F, Zhang P, An XM, et al. CD4+CD25+ regulatory T cells decreased the antitumor activity of cytokine-induced killer (CIK) cells of lung cancer patients. *J Clin Immunol* 2007;27:317-26.
- Pritchard J, Makowska A, Gregson NA, Hayday AC, Hughes RA. Reduced circulating CD4+CD25+ cell populations in Guillain-Barré syndrome. *J Neuroimmunol* 2007;183:232-8.
- Keever-Taylor CA, Browning MB, Johnson BD, Truitt RL, Bredeson CN, Behn B, et al. Rapamycin enriches for CD4(+) CD25(+) CD27(+) Foxp3(+) regulatory T cells in ex vivo-expanded CD25-enriched products from healthy donors and patients with multiple sclerosis. *Cytotherapy* 2007;9:144-57.
- Kinter AL, Horak R, Sion M, Riggan L, McNally J, Lin Y, et al. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 2007;23:438-50.
- Hallermann C, Niermann C, Schulze HJ. Regulatory T-cell phenotype in association with large cell transformation of mycosis fungoides. *Eur J Haematol* 2007;78:260-3.
- Shi HZ, Li S, Xie ZF, Qin XJ, Qin X, Zhong XN. Regulatory CD4+CD25+ T lymphocytes in peripheral blood from patients with atopic asthma. *Clin Immunol* 2004;113:172-8.
- Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Rückert B, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* 2004;114:1425-33.
- Xystrakis E, Kusumakar S, Boswell S, Peek E, Urry Z, Richards DF, et al. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Invest* 2006;116:146-55.
- Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007;119:1258-66.
- Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin Exp Immunol* 2007;148:53-63.
- National Institutes of Health. Global Strategy for Asthma Management and Prevention. National Institutes of Health National Heart, Lung and Blood Institute report. Bethesda (MD): National Institutes of Health; 2002. p. 67-79. NIH publication no. 02-3659.
- Masi AT, Hunder GG, Lie JT, Michel BA, Bloch DA, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis). *Arthritis Rheum* 1990;33:1094-100.
- Landay AL, Muirhead KA. Procedural guidelines for performing immunophenotyping by flow cytometry. *Clin Immunol Immunopathol* 1989;52:48-60.
- Elson LH, Nutman TB, Metcalfe DD, Prussin C. Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2 and T helper 0 cells within the human CD4+CD27- lymphocyte subpopulation. *J Immunol* 1995;154:4294-301.
- Watts RA, Carruthers DM, Scott DG. Epidemiology of systemic vasculitis: changing incidence or definition? *Semin Arthritis Rheum* 1995;25:28-34.
- Martin RM, Wilton LV, Mann RD. Prevalence of Churg-Strauss syndrome, vasculitis, eosinophilia and associated conditions: retrospective analysis of 58 pre-scription-event monitoring cohort studies. *Pharmacoepidemiol Drug Saf* 1999;8:179-89.
- Tsurikisawa N, Morita S, Tsuburai T, Oshikata C, Ono E, Saito H, et al. Familial Churg-Strauss syndrome in two sisters. *Chest* 2007;131:592-4.
- Tsurikisawa N, Tsuburai T, Saito H, Morita S, Horiguchi Y, Mitomi H, et al. A retrospective study of bronchial hyperresponsiveness in patients with asthma before the onset of Churg-Strauss syndrome. *Allergy Asthma Proc* 2007;28:336-43.
- Tsurikisawa N, Taniguchi T, Saito H, Himeno H, Ishibashi A, Suzuki S, et al. Treatment of Churg-Strauss syndrome with high-dose intravenous immunoglobulin. *Ann Allergy Asthma Immunol* 2004;92:80-7.
- Pavone L, Grasselli C, Chierici E, Maggiore U, Garini G, Ronda N, et al. Outcome and prognostic factors during the course of primary small-vessel vasculitides. *J Rheumatol* 2006;33:1299-306.
- Astier AL, Meiffren G, Freeman S, Hafler DA. Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J Clin Invest* 2006;116:3252-7.
- Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3+CD25+CD4+regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 2005;201:723-35.
- Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J Immunol* 2007;178:2018-27.