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Original Article

Role of new population of peripheral CD11c$^+$CD8$^+$ T cells and CD4$^+$CD25$^+$ regulatory T cells during acute and remission stages in rheumatoid arthritis patients

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Background and Purpose: Rheumatoid arthritis (RA) is a CD4$^+$-dependent chronic systemic inflammatory disease with autoimmune features. Autoreactive CD4$^+$ T-cell activation can result in autoimmune diseases. One of the key regulators is the CD4$^+$CD25^{high} regulatory T (Treg) cell. In an animal arthritis model, CD11c$^+$CD8$^+$ T cells were found to be elevated, and could suppress pathogenic CD4$^+$ T cells after cross-linking with CD137. The purpose of this study was to compare the expression of CD137, CD4$^+$CD25^{high} Treg cells, and CD11c$^+$CD8$^+$ in the peripheral blood T lymphocytes of RA patients during active and remissive states, and evaluate the correlation with disease activity.

Methods: Thirty nine RA patients treated at the rheumatology outpatient clinic at the Changhua Christian Hospital were assessed clinically for disease activity and classified as either highly active or remissive by the Disease Activity Score 28. Peripheral blood mononuclear cells were isolated from these patients and compared against normal controls.

Results: The presence of CD11c$^+$CD8$^+$ T cells or the expression of CD137 molecules in peripheral blood cells was not related to disease activity. In contrast, CD4$^+$CD25^{high} Treg cell levels were increased significantly in patients with active RA compared with patients with remissive RA or controls (p<0.05). These lymphocytes were intact, without evidence of apoptosis.

Conclusions: Our results indicate that CD4$^+$CD25^{high} Treg cells play an important role in modulating RA disease activity and can serve as a parameter of disease activity.

Key words: Apoptosis; Arthritis, rheumatoid; CD4-positive T-lymphocytes; CD8-positive T-lymphocytes; T-Lymphocytes, regulatory

Introduction

Rheumatoid arthritis (RA) is a CD4$^+$-dependent chronic systemic inflammatory disease with autoimmune features [1]. The prevalence of RA in Taiwan is approximately 5%. RA is characterized by chronic synovial inflammation resulting in cartilage and bone damage, eventually leading to joint destruction. Several different cell types such as T cells, B cells, macrophages, and their mediators, are involved in the tissue-destructive inflammation [2,3]. Disturbed immune regulation resulting in T-helper type 1 (Th1)/Th2 cell imbalances, and a continuous pathogenic activation of T cells was suggested as a cause of chronic inflammation of RA.

Autoreactive T cells capable of recognizing tissue-specific antigens escape negative selection. These cells appear in the human blood circulation and can be cloned from the lymph nodes of mice. Their activation can result in autoimmune disease. The immune system has developed several mechanisms to suppress or regulate immunity in order to protect the body from a sustained, harmful immune response [4]. One of the

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key players of immune regulation is the $CD4^+CD25^+$ regulatory T (Treg) cell. A number of experimental studies have shown that $CD4^+CD25^+$ Treg cells can inhibit autoimmune diabetes [5] and prevent inflammatory bowel disease [6]. All subsegment in vitro studies indicate that $CD4^+CD25^+$ Treg cells are involved in the suppression of the autoimmune response [7,8] and are part of the normal human immune response [9,10]. $CD4^+CD25^+$ Treg cells can prevent both the activation and effective functioning of autoreactive T cells, which escape the mechanism of tolerance [9-12]. However, only cells expressing the highest levels of CD25 ($CD4^+CD25^{high}$) demonstrate potent regulatory functions [13-15]. The first aim of our study was to investigate whether the proportion of $CD4^+CD25^{high}$ Treg cells in peripheral blood was different in active and remissive RA patients.

Costimulatory signaling can condition T cells to develop into diverse effector cells. In addition to CD28-CD80 or CD86, which provide positive signals for initial T-cell activation [16,17], a number of other receptor ligand pairs may be involved in sustaining, diversifying, and/or amplifying the immune response. In recent years, CD137 (4-1BB), a member of the tumor necrosis factor receptor (TNFR)/tumor necrosis factor (TNF) ligand family, was found to be important in regulating T cell responses subsequent to initial activation, and to be expressed on activated CD4 T cells, CD8 T cells, and activated antigen-presenting cells, including B cells, macrophages, and dendritic cells (DCs) [18-20]. TNFR has been shown to inhibit the function of Treg cells that control immune effector cells. Treating Treg cells with specific antibodies to TNFR affects the suppressive action of Treg cells in vitro and in autoimmune animal models such as systemic lupus erythematosus and arthritis. Indeed, CD137 costimulation could inhibit or prevent established disease [21-24]. The second purpose of this study was to investigate whether the expression of CD137 was different in active and remissive RA patients.

A new population of lymphocytes, $CD11c^+CD8^+$ T cells, was identified in collagen type II-induced arthritis studies as a result of anti-CD137 monoclonal antibody (mAb) administration with collagen type II. It has been suggested that cross-linking of CD137 leads to the expansion of $CD11c^+CD8^+$ T cells that produce interferon-gamma, the paracrine effects of which, in turn, regulate the transcription of the indoleamine 2,3-dioxygenase gene in DCs and macrophages [23]. Through an indoleamine 2,3-dioxygenase-dependent mechanism, $CD11c^+CD8^+$ T cells consequently suppress pathogenic $CD4^+$ T cells without global immunosuppression.

The third purpose of this study was to investigate new populations of $CD11c^+CD8^+$ T cells during active and remissive stages in RA patients. These findings were correlated with clinical features to determine whether differences in these regulatory cell populations between the different subtypes of RA can explain differences in clinical features.

Methods

Patient population

From December 2005 to June 2006, 39 RA patients who were being regularly treated in the rheumatology outpatient clinic at the Changhua Christian Hospital were enrolled in the study. All patients were identified by the 1987 American Rheumatism Association (ARA) criteria. RA patients were excluded from this study according to the following criteria: 1) patients who were under 15 or older than 75 years of age; 2) patients who received treatment with Enbrel (etanercept) or infliximab; and 3) pregnant women.

The Disease Activity Score 28 (DAS28) was used to evaluate the current activity of RA and information on variable diseases needed for calculations.

A DAS28 >5.1 reflects high disease activity whereas a DAS28 <3.2 indicates low disease activity. Remission is shown by a DAS28 <2.6 (comparable to the ARA remission criteria).

All RA patients were assessed clinically for disease activity and classified either as highly active or remissive. Patients with a DAS28 >5.1, an erythrocyte sedimentation rate over 50 mm/h, a joint swollen with synovial fluid accumulation, and joint tenderness were classified in the high activity group. Patients with a DAS28 <3.2, an erythrocyte sedimentation rate below 25 mm/h, followed up in the past 6 months, and without swollen joints or synovial fluid accumulation were classified in the remission group. All patients received continuous follow-up in our out-patient clinic.

Peripheral blood mononuclear cells (PBMCs) were isolated from 6 mL of heparinized venous blood using a differential centrifugation gradient (Ficoll-Hypaque; Amersham Pharmarcia Biotech, Piscataway, NJ, USA). PBMCs were isolated and analyzed immediately when all blood samples were obtained. Healthy adult volunteers were selected as a control group. Both study and control groups were age- and sex-matched to the

extent possible. The study was performed with the approval of the Institutional Review Board at the Changhua Christian Hospital and informed consent was obtained from each patient.

Cell apoptosis and cellular staining procedure

Annexin V conjugated to a fluorochrome, fluorescein isothiocyanate (FITC), was used for flow cytometric identification of peripheral blood lymphocytes (PBLs) in the early stages of apoptosis. The translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is one of the earliest indications of apoptosis. Annexin V, a phospholipid-binding protein with a high affinity for PS, recognizes the exposed binding sites on PS. In addition, cell surface markers for PBLs were labeled with anti-CD4 (PC7) mAb, anti-CD8a (PC5) mAb, anti-CD11c (PE) mAb, and anti-CD137 (FITC) mAb simulateously (BioLegend, San Diego, CA, USA). After staining, cells were analyzed by fluorescence-activated cell sorter scan flow cytometry (FC500; Beckman Coulter Inc., Fullerton, CA, USA) acquiring 10,000 events.

A typical CD25 expression profile is shown in Fig. 1, together with a dot plot used to count the $CD4^{+}CD25^{high}$ cells.

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences for Windows (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation. Differences between means in each group were analyzed by analysis of variance, followed

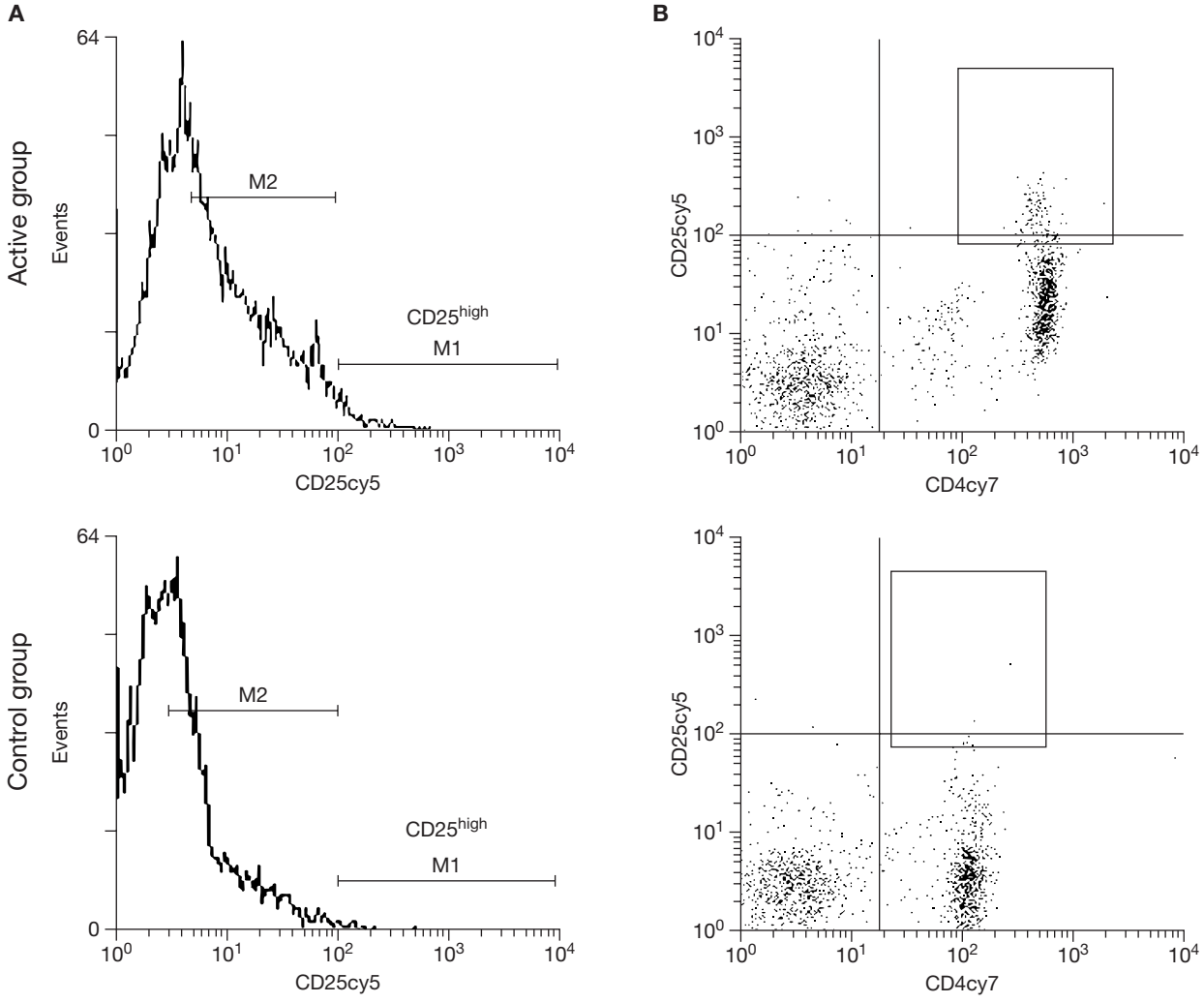

Fig. 1. Analysis of cell surface markers by flow cytometry. (A) Single-parameter histogram indicating CD25 expression in peripheral blood mononuclear cell subsets; (B) dot plot showing CD25 expression vs CD4 expression; cells within the solid box are $CD4^{+}CD25^{high}$ cells. M1 = the region of $CD4^{+}CD25^{high}$ in the histogram; M1 + M2 = the region of $CD4^{+}CD25^{+}$ in the histogram.

by the Tukey's test. A p value <0.05 was considered significant.

Results

Clinical features

A total of 39 RA patients (29 women and 10 men) were enrolled in this study. Nineteen patients were included in the active disease group and 20 patients were included in the remission group. Nine subjects without rheumatic disease, without other autoimmune diseases, and with normal serum rheumatoid factor immunoglobulin M levels were selected as healthy control subjects. None of the female patients were pregnant. The baseline characteristics of the active, remission, and control groups are listed in Table 1.

The mean (± standard deviation) ages of the active, remission, and control groups were 58.5 ± 11.52, 50.60 ± 14.67, and 37.78 ± 13.65 years, respectively. The mean DAS28 in the active and remission groups were 5.87 ± 0.68 and 2.26 ± 0.60, respectively. The active disease group had more frequent arthritis in 3 or more joints, as well as arthritis in hand joints (independent samples t test, p<0.05).

Correlation between disease activity and CD4$^+$CD25^{high} Treg cells

To study whether CD4$^+$CD25$^+$ Treg cells were increased during the active phase of RA, we conducted a simultaneous flow cytometric study in the same permeabilized cells with surface markers and terminal deoxynucleotidyl-mediated deoxyuridine triphosphate nick end labeling expression. We found a significant increase in the percentage of the total CD4$^+$CD25$^+$ Treg cells in the active RA group when compared to the controls (15.78 ± 10.04% vs 4.90 ± 3.45%; p=0.003). Similar results were obtained for CD4$^+$CD25^{high} T cells, as shown in Fig. 2 (1.49 ± 1.57% vs 0.20 ± 0.25%; p=0.024). The regression equation from the scatter plot of CD4$^+$CD25^{high} Treg cells vs DAS28 was: CD4$^+$CD25^{high} Treg cells = 0.15 + 0.24 × DAS28; R^2 = 0.17 (Fig. 3). However, there was no statistical difference in the percentage of CD4$^+$CD25^{high} T cells expressing CD4$^+$CD25$^+$ cells among each group (12.43 ± 21.42%, 7.40 ± 5.54% vs 4.00 ± 3.18%; p=0.3).

Correlation between disease activity, CD137, and CD11c$^+$CD8$^+$

CD137 is a member of the TNFR/TNF family and has inhibitory effects on Treg cells. Cross-linking of CD137 leads to the production of interferon-gamma in CD11c$^+$CD8$^+$ T cells. We found that there was no significant difference in CD137 expression on peripheral lymphocytes among the 3 groups of RA patients (Fig. 4) [p=0.754]. Moreover, the expression of CD137 on the CD8$^+$ T cells or CD11c$^+$ cells was not statistically different (p=0.64, p=0.48). The population of CD11c$^+$ CD8$^+$ T cells in healthy adults was approximately 10%

Table 1. Clinical characteristics of the 3 groups

Characteristics	Active group (n = 19) No. (%)	Remission group (n = 20) No. (%)	Control group (n = 9) No. (%)
Age (years; mean ± SD)	58.5 ± 11.52	50.60 ± 14.67	37.78 ± 13.65
Gender ratio (male:female)	4:15	6:14	5:4
Symmetric arthritis	17 (94.4)	9 (52.9)	-
Arthritis in 3 or more joints	16 (88.9)	2 (11.8)	-
Arthritis of hand joints	16 (88.9)	3 (17.6)	-
Rheumatoid factor-positive	15 (83.3)	13 (72.7)	-
Radiographic changes			
Symmetric polyarticular disease of synovial joints	13 (72.2)	9 (56.3)	-
Soft tissue swelling	15 (83.3)	12 (75.0)	-
Periarticular osteoporosis	14 (77.8)	13 (81.3)	-
Marginal erosions; cysts	15 (83.3)	12 (75.0)	-
Diffuse loss of joint space	12 (66.7)	10 (62.5)	-
Extra-articular features			
Anemia	1 (6.3)	1 (6.3)	-
Sicca syndrome	1 (6.3)	1 (6.3)	-
Leukopenia (<4.0 × 10^9/L)	1 (1.3)	1 (6.3)	-
Renal involvement	-	12 (11.1)	-

Abbreviation: SD = standard deviation

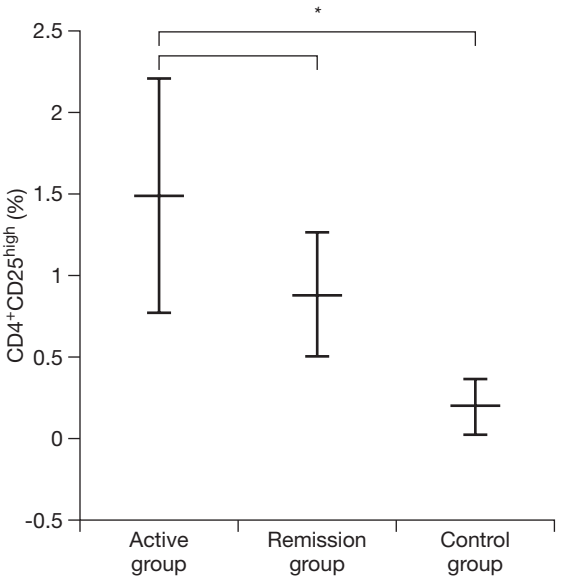

Fig. 2. Percentage of $CD4^+CD25^{high}$ T lymphocytes after gating by flow cytometry from the active rheumatoid arthritis, remission rheumatoid arthritis, and control groups. *Mean $p<0.05$.

in this study. Although there was no significant statistical difference in the number of $CD11c^+CD8^+$ cells between the groups ($p=0.157$), a notable difference was observed between the groups when the percentage of $CD11c^+CD8^+$ in $CD8^+$ T cells were compared ($p=0.078$) [Fig. 5]. There was no correlation between the CD137 and $CD11c^+CD8^+$ cell populations, which contradicted our supposition. The regression equation was: $CD11c^+CD8^+ = 13.47 + 0.07 \times CD137$; $R^2 = 0.02$. The correlation between

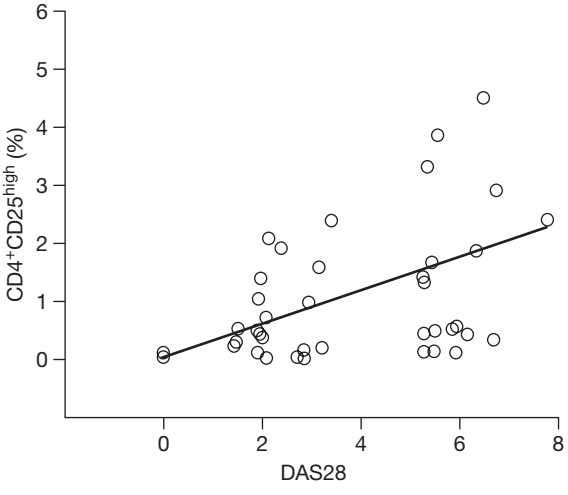

Fig. 3. Scatter plot of the percentage of peripheral $CD4^+CD25^{high}$ T lymphocytes vs Disease Activity Score 28 (DAS28) in rheumatoid arthritis patients. The regression equation from the scatter plot is: $CD4^+CD25^{high}$ Treg cells = $0.15 + 0.24 \times$ DAS28; $R^2 = 0.17$. Treg = regulatory T.

$CD4^+CD25^{high}$ Treg cells and $CD11c^+CD8^+$ cells was also poor. The regression equation was: $CD11c^+CD8^+ = 16.59 - 0.40 \times CD4^+CD25^{high}$ Treg cells; $R^2 = -0.001$.

Apoptosis of lymphocytes

To determine whether apoptosis of $CD4^+CD25^{high}$ Treg cells or $CD11c^+CD8^+$ cells occurred in active RA, a simultaneous study was performed. All PBLs were negative for annexin V among the active, remission, and control groups. PS translocation had not occurred and the plasma membrane was still intact. This indicated that cells were still viable with no evidence of apoptosis.

Discussion

Many groups have monitored the frequency of Treg cells in the peripheral blood of patients with RA, with varying conclusions. Differences in therapy and duration of disease are 2 possible explanations. Many patients in this study received treatment with disease-modifying antirheumatic drugs (DMARDs) for several years. The report from van Amelsfort et al was in accordance with our data, showing a significant increase in total $CD4^+CD25^+$ T cells in patients with RA receiving DMARD regimens compared with controls [25]. On the contrary, Cao et al [26] showed that there was no statistical difference in the frequency of total $CD4^+CD25^+$ T cells between RA and control patients, but they found that the $CD4^+CD25^{high}$ population of T cells was reduced in RA patients years later [27]. Other groups also did not detect a statistically significant difference in the frequency of $CD4^+CD25^{high}$ T cell between active RA patients receiving DMARD regimens and healthy controls [28-30].

Lawson et al [31] demonstrated that in patients with early RA, there was a deficit in the $CD4^+CD25^{high}$ Treg cell population in peripheral blood. Recently, Sutmuller et al [32] found that the Toll-like receptor 2 controls the expansion and function of Treg cells. Moreover, when combined with T cell receptor stimulation, Toll-like receptor 2 triggering augmented Treg proliferation in vitro and in vivo, and resulted in a temporal loss of the suppressive Treg phenotype in vitro by directly affecting Treg. Whether a similar mechanism results in a higher number of Treg cells but not in increased function of these cells in our patients needs further study.

Using an animal arthritis model, Seo et al formulated the theory that cross-linking of CD137 leads to the expansion of $CD11c^+CD8^+$ T cells, which in turn

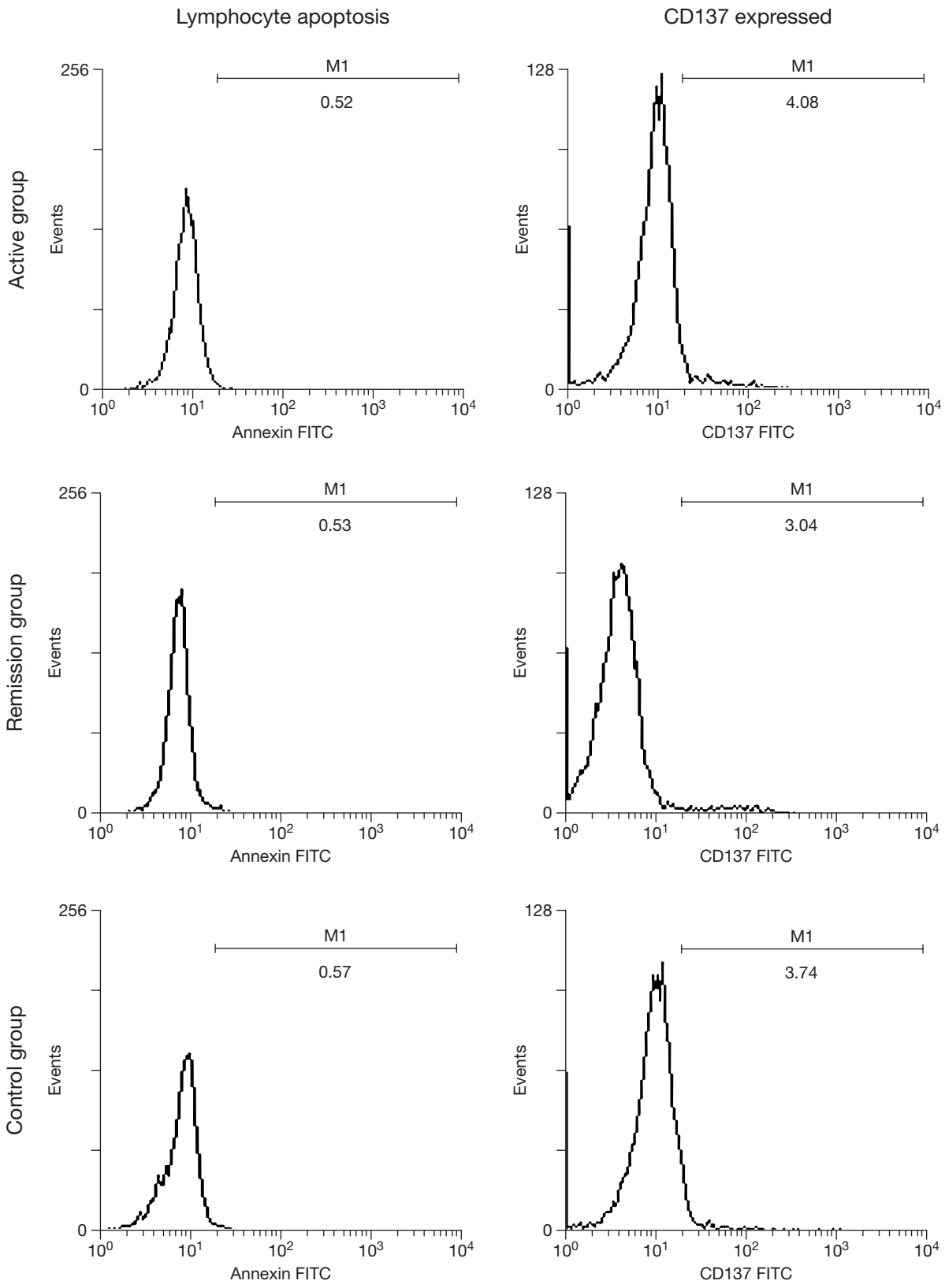

Fig. 4. Peripheral lymphocyte apoptosis and CD137 expression in the active rheumatoid arthritis, remission rheumatoid arthitis, and control groups. Numbers above each histogram indicate the percentage of cells stained. FITC = fluorescein isothiocyanate. M1 = the expressing region of annexin V and CD137.

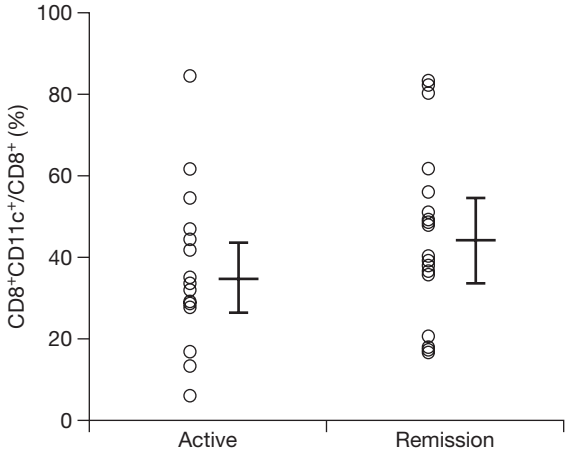

Fig. 5. Percentage $CD11c^+CD8^+$ expression in $CD8^+$ T cells in the active rheumatoid arthritis and remission rheumatoid arthritis groups (p=0.078 between groups).

suppresses pathogenic $CD4^+$ T cells by regulating DCs and macrophages [23]. Our results, however, did not show any correlation between disease activity and peripheral $CD11c^+CD8^+$ blood T cells (R^2 = 0.001). Furthermore, the results indicated a poor correation between the percentages of $CD137^+$ and $CD11c^+CD8^+$ cells in the peripheral blood.

Balanescu et al reported that clinically active RA (DAS28>5.1) was associated with a high incidence of activated DCs in the synovial tissue and synovial fluid, but found no difference between circulating DCs in RA patients and controls [33]. In contrast, our results suggested that $CD8^+$ PBLs might actually enhance the $CD11c^+$ surface marker in RA patients, but this was not related to disease activity. Based on the proposal by Seo et al [23] that the immune modulation performed by $CD11c^+CD8^+$ T cells was not associated with global immunosuppression, and also the poor correlation found between $CD11c^+CD8^+$ T cells and disease activity in our studies, we speculated that costimulation of CD137 in $CD11c^+CD8^+$ T cells might be important in regulating local inflammation, as in synovial tissue, rather than systemic inflammation.

Animal studies have shown that CD137 expression on activated T cells is transient, and conditions CD4 cells early on but not after differentiation. Most studies regarding 4-1BB (CD137) expression suggest that 4-1BB is expressed on activated cells at 24 h after activation, with peak expressions at 48-72 h [34,35]. The in vivo expression of 4-1BB was examined following superantigen administration, and expression was found to be more rapid and transient than observed in vitro (female B10.A mice) [36]. These characteristics were confusing with regards to the adequate blood sampling time, possibly confounding the calculation of the time between disease flaring and blood isolation. The cell numbers of each lymphocyte subgroup changed greatly within 2 h of blood isolation from patients. Although all blood samples were analyzed within 1 h in this study, it was still a challenge to decide the datum point of comparison among patients. Michel et al reported that soluble CD137 is enhanced in the sera of patients with RA [37]. If a good correlation was established, serum soluble CD137 level could substitute for surface CD137 as a more accurate measurement.

Maerten et al's mouse model showed that $CD4^+$ $CD25^+$ Treg cells obtained from 4-1BB-deficient mice were able to prevent naive CD4+ T cell-induced colitis. Maerten et al concluded that 4-1BB and 4-1BB ligand interaction modulates the effector CD4 T cell-driven immune response without affecting Treg cell function [38]. Our results also showed that peripheral blood $CD4^+CD25^{high}$ Treg cells play a role in affecting disease activity and account for approximately 20% of the variability (R^2 = 0.17). Furthermore, these results seem compatible with Maerten et al's finding that CD137 and $CD4^+CD25^+$ can affect disease activity via different mechanisms or even at different locations.

Apoptosis of Th2 lymphocytes in asthmatic children treated with immunotherapy was found in a recent study [39]. However, Th2 cell apoptosis was not found in the present study. Our data provide evidence that $CD4^+CD25^+$ T cells in RA patients might be involved in immune regulation but not in inducing apoptosis.

In conclusion, $CD11c^+CD8^+$ T cells and the CD137 molecule in peripheral blood do not affect disease activity in RA patients, and there is a weak relationship between $CD11c^+CD8^+$ T cells and CD137 expressed in peripheral blood T cells in these patients. $CD4^+CD25^{high}$ Treg cells modulate systemic inflammation by regulating the immune response, but not by inducing apoptosis in RA patients. RA patients who receive DMARD therapy may show an increase of $CD4^+CD25^{high}$ cells when the disease flares, but the proliferation of functional $CD4^+CD25^{high}$ is not increased.

In contrast, a significant increase in $CD4^+CD25^{high}$ Treg cells was observed in patients with active RA compared to those in remissive RA or control subjects. These lymphocytes were intact without apoptosis. These results indicate that $CD4^+CD25^{high}$ Treg cells play an important role in the modulation of active disease and can serve as a parameter of disease activity.

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