EXTENDED REPORT

Predominance of Th1 cytokine in peripheral blood and pathological tissues of patients with active untreated adult onset Still's disease

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Objective: To determine the type 1 T helper (Th1)/type 2 T helper (Th2) balance in the peripheral blood (PB) and pathological tissues of patients with active untreated adult onset Still's disease (AOSD).
Methods: The percentages of interferon γ (IFNγ)- and interleukin (IL)4-producing Th cells in the PB of 20 patients with active untreated AOSD, 20 patients with active rheumatoid arthritis (RA), and 20 healthy controls were determined by intracellular staining and flow cytometry. Serum levels of IL18 and soluble IL2 receptor were measured by enzyme linked immunosorbent assay. Levels of IFNγ and IL4 messenger (m) RNA expression were examined by real time quantitative polymerase chain reaction in biopsy specimens of evanescent rash and synovitis from 8 patients with AOSD.
Results: Significantly higher IFNγ-producing Th cells and Th1/Th2 ratio in PB were found in patients with AOSD than in healthy controls. Percentages of IFNγ-producing Th cells and Th1/Th2 ratio in PB correlated significantly with clinical activity score and serum IL18 levels in patients with AOSD. Increased ratio of Th1/Th2 cytokine transcripts was seen in the biopsy specimens of evanescent rash and synovitis from patients with AOSD compared with normal skin controls and patients with OA. Th cell cytokine pattern in PB and cytokine mRNA expression in synovium were similar for patients with AOSD and with RA. After 3 months' treatment, clinical remission was associated with a marked decrease in the percentages of cytokine-producing Th1 cells, but not of the Th2 cells.
Conclusion: A predominance of Th1 cytokine may precipitate the pathogenesis of AOSD.

Adult onset Still's disease (AOSD) is an inflammatory disorder characterised by spiking fever, evanescent skin rash, arthralgia or arthritis, sore throat, and hepatosplenomegaly, as well as by laboratory abnormalities, which include neutrophilic leucocytosis, abnormal liver function tests, and raised levels of serum ferritin and acute phase reactants.[1][2] It is considered to be identical to the systemic form of juvenile rheumatoid arthritis (JRA).[1] The aetiopathogenesis of this disease remains unclear. Recently, alterations in cytokine production have been suggested as a possible important pathogenic factor and as a potential target for therapeutic intervention in autoimmune disease.[3] Previous studies have indicated that interleukin (IL)6 is a useful marker of disease activity in patients with AOSD.[4][5] Hoshino et al also found that serum levels of IL6, tumour necrosis factor α, and interferon γ (IFNγ) were significantly increased in 12 patients with active AOSD compared with healthy controls.[5] A recent study has shown that serum IFNγ levels are raised in patients with active AOSD, and that serum levels of IL18 correlate with serum ferritin values and disease severity, suggesting that IL18 has a central role in the pathogenesis of this disease.[6] Fujii et al reported that serum levels of soluble IL2 receptor (sIL2R), which reflects T cell activation,[7] IL4, and IL18 correlated with disease activity or C reactive protein level in chronic articular AOSD.[8]

Valente et al recognised a distinct paracortical hyperplasia of the lymph node biopsies in seven of eight patients with AOSD, providing indirect evidence of a T cell mediated disease.[9] Further, an immunohistological study of a lymph node biopsy from one patient with AOSD showed exclusive proliferation of T cells, consisting mainly of T helper (Th) cells.[10] These findings suggest that Th cells may play a critical part in the pathogenesis of AOSD. Th cells, which are central to the immune process, have been classified into two

functional subsets according to their cytokine expression patterns and separate effector functions.[11–13] Type 1 Th (Th1) cells, which produce mainly IFNγ and IL2, promote cell mediated immunity; whereas type 2 Th (Th2) cells, which secrete IL4, IL5, IL10, and IL13, are associated with humoral immune response. It has been demonstrated that the Th1/Th2 cytokine imbalance has a key role in the induction and development of several autoimmune diseases.[14] Correction of this imbalance has become the new therapeutic strategy for animal models with allergic encephalomyelitis, which is a prototypic autoimmune disease produced by Th1-like T cells.[15] Although previous studies have noted deregulated serum or plasma cytokine production in patients with AOSD, there are no data for the percentages of Th1 and Th2 cytokines in a defined cell population from peripheral blood (PB) samples of patients with this disease.

In this study Th1 and Th2 cells were quantified at the single cell level in PB of patients with active untreated AOSD and patients with active rheumatoid arthritis (RA) using intracellular cytokine staining and flow cytometry to determine Th cell polarisation in these two diseases. We chose patients with RA as disease controls because a number of previous studies have documented the Th cytokine pattern in this disease.[16][17] The serum levels of IL18, which is a new cytokine with pleiotropic activities critical to the development

of Th1 responses,[18] were also evaluated for patients with active untreated AOSD and patients with active RA by enzyme linked immunosorbent assay (ELISA). The correlations between the percentage of cytokine-producing Th cells or the Th1/Th2 ratio and clinical activity score and serum IL18 levels were also investigated in our patients with AOSD. To clarify the in vivo Th1/Th2 imbalance in patients with AOSD, we analysed the mRNA expression of Th1 (IFNγ) and Th2 (IL4) cytokines in biopsy specimens of evanescent rash and synovitis from eight patients with active untreated AOSD by a real time quantitative polymerase chain reaction (RQ-PCR). Changes in the levels of IL18 and sIL2R in serum, and in the percentages of cytokine-producing Th cells and the Th1/Th2 balance in PB during longitudinal follow up of patients with treated AOSD were also studied.

PATIENTS AND METHODS
Patients
Twenty patients (15 women and five men, mean (SD) age 36.0 (14.9) years) with active untreated disease, fulfilling the Yamaguchi criteria for AOSD,[19] were enrolled. Patients with infections, malignancies, or other rheumatic diseases were excluded. The clinical activity score for each patient with AOSD was assessed as described by Rooney.[20] After AOSD diagnosis and the initial investigation, all patients were treated with glucocorticosteroids (GC), non-steroidal anti-inflammatory drugs (NSAIDs), and oral methotrexate (MTX). All patients with AOSD achieved complete or partial remission within 3 months of the start of treatment. Complete remission was defined as the total absence of nine clinical symptoms: high fever, typical rash, articular manifestations, myalgia, sore throat, lymphadenopathy, hepatomegaly, splenomegaly, and serositis. If any one of these symptoms persisted after treatment, the remission was considered partial. Twenty healthy volunteers matched for age were used as normal controls (18 women and two men, mean (SD) age 32.4 (10.0) years). Twenty patients with active classic RA (19 women and one man, mean (SD) age 51.2 (11.8) years) were included in this study as disease controls. Inclusion criteria were fulfilment of the 1987 revised criteria of the American College of Rheumatology[21]; disease duration over 1 year; and, active arthritis, defined as more than six swollen and six tender joints for at least 3 months. All patients with RA received low doses of oral prednisolone (5–10 mg daily), NSAIDs, and no disease modifying anti-rheumatic drugs, except for oral MTX 12.5–17.5 mg weekly, at least 1 month before enrolment in the study. PB was collected using endotoxin-free heparinised vacuum tubes (KABI-ET; Chromogenix, Antwerp, Belgium) to avoid cytokine production during the interval between sampling and culture. Study protocol was approved by the ethics committee of clinical research at Taichung Veterans General Hospital, and informed consent was obtained from each participant.

Th1/Th2 cytokine analysis with flow cytometry for peripheral whole blood
Flow cytometry analysis for intracellular cytokines (IFNγ and IL4) was performed with slight modification according to the described technique.[22] Briefly, aliquots of 500 μl of sterile heparinised whole blood were stimulated with a combination of 25 ng/ml of phorbol myristate acetate (PMA) and 1 μg/ml of ionomycin (Sigma, Germany) in the presence of 10 μg/ml of brefeldin A (Sigma) and cultured for 4 hours at 37°C in a humidified 5% CO_2 incubator. Activated cultures of blood samples were aliquoted and stained with 20 μl of peridinin chlorophyll protein, cyanin 5.5 conjugated CD3- and CD4-specific monoclonal antibody (mAb) (Becton Dickinson, USA) for 15 minutes at room temperature. Erythrocytes were lysed by adding 2 ml of fluorescence activated cell sorter

lysing solution (Becton Dickinson). After 5 minutes' incubation, samples were centrifuged and washed with 0.1% bovine serum albumin-phosphate buffered saline (BSA-PBS), and subsequently incubated with the fluorescence activated cell sorter permeabilising solution, saponin (Becton Dickinson), for 10 minutes at room temperature in the dark. The samples were washed twice with 0.1% BSA-PBS, and incubated with fluorescein isothiocyanate conjugated IFNγ-specific mAb and phycoerythrin conjugated IL4-specific mAb (Becton Dickinson) for 30 minutes at room temperature in the dark. Surface and intracytoplasmic phycoerythrin conjugated CD69 staining was measured in PMA + ionomycin and PMA + ionomycin + saponin activated T cells, respectively, to ensure activation and permeabilisation efficiency. After washing, the cells were resuspended in 1% paraformaldehyde and analysed by two colour flow cytometry (FACScan; Becton Dickinson). Controls were introduced at each of three steps of staining specificity, activation, and permeabilisation. Data were obtained using a FACScan flow cytometer, and the results analysed using Cellquest software (Becton Dickinson).

RNA extraction and RQ-PCR in biopsy specimens of evanescent rash and synovitis from patients with active untreated AOSD
Skin specimens were taken from eight patients with evanescent Still's rash and from four healthy people undergoing plastic surgery. Synovial membrane (SM) was taken from six patients with AOSD with active synovitis, six patients with active RA, and four patients with osteoarthritis (OA). The biopsy specimens were homogenised in 500 μl RNAzol B solution on ice using a homogeniser. Total cellular RNA was isolated from biopsy tissues by the guanidinium isothiocyanate method,[23] extracted with phenol/chloroform, precipitated with isopropanol, washed in 70% ethanol, and dissolved in diethylpyrocarbonate treated, double distilled water. RNA was measured by spectrophotometry at 260 nm. A 2.5 μg aliquot was reverse transcribed with 200 U of Moloney murine leukaemia virus reverse transcriptase according to standard procedures (Boehhringer Ingelheim).

IFNγ and IL4 mRNA expression was quantified by real time TaqMan PCR according to the manufacturer's instruction (Corbette Research, Mortlake, NSW, Australia). The design of the sequence-specific probe (TaqMan probe), combined with the 5′-nuclease activity of the Taq DNA polymerase, allows for the direct detection of the PCR products by the release of a fluorescent reporter during PCR on the Rotor-Gene 2000 thermal real time cycler (Corbette Research, Mortlake, NSW, Australia). According to the manufacturer, a given reaction in a tube is calculated by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference and is defined as normalised reporter Rn. The value of a reaction containing all components including template minus the value of a reaction containing samples without template is the value of ΔRn. The C_T values (threshold cycles) are cycles at which statistically significant increases in ΔRn are first detected and are selected in the line in which all samples are in logarithmic phase.[24] Threshold is defined as the average standard deviation of Rn for the early cycles, multiplied by an adjustable factor. The following oligonucleotide primers and TaqMan probes for IFNγ and IL4 were designed and synthesised. For IFNγ, sense primer 5′-TGCAGAGCCAAATTGTCTCC-3′ and anti-sense primer 5′-AACTGTAAGTACAGAAGGAA -3′, TaqMan probe 5′-ATGACCAGAGCATC CAAAA-3′; and for IL4, sense primer 5′-GATCGTTAGCTTCTCCTGATAAAC-3′ and anti-sense primer 5′-AACCCTCCACTCTGGGTAATT-3′, TaqMan probe 5′-CCTCACATT GTCACTGCAAA-3′. The TaqMan probe consists of an oligonucleotide with a 5′ reporter dye and a
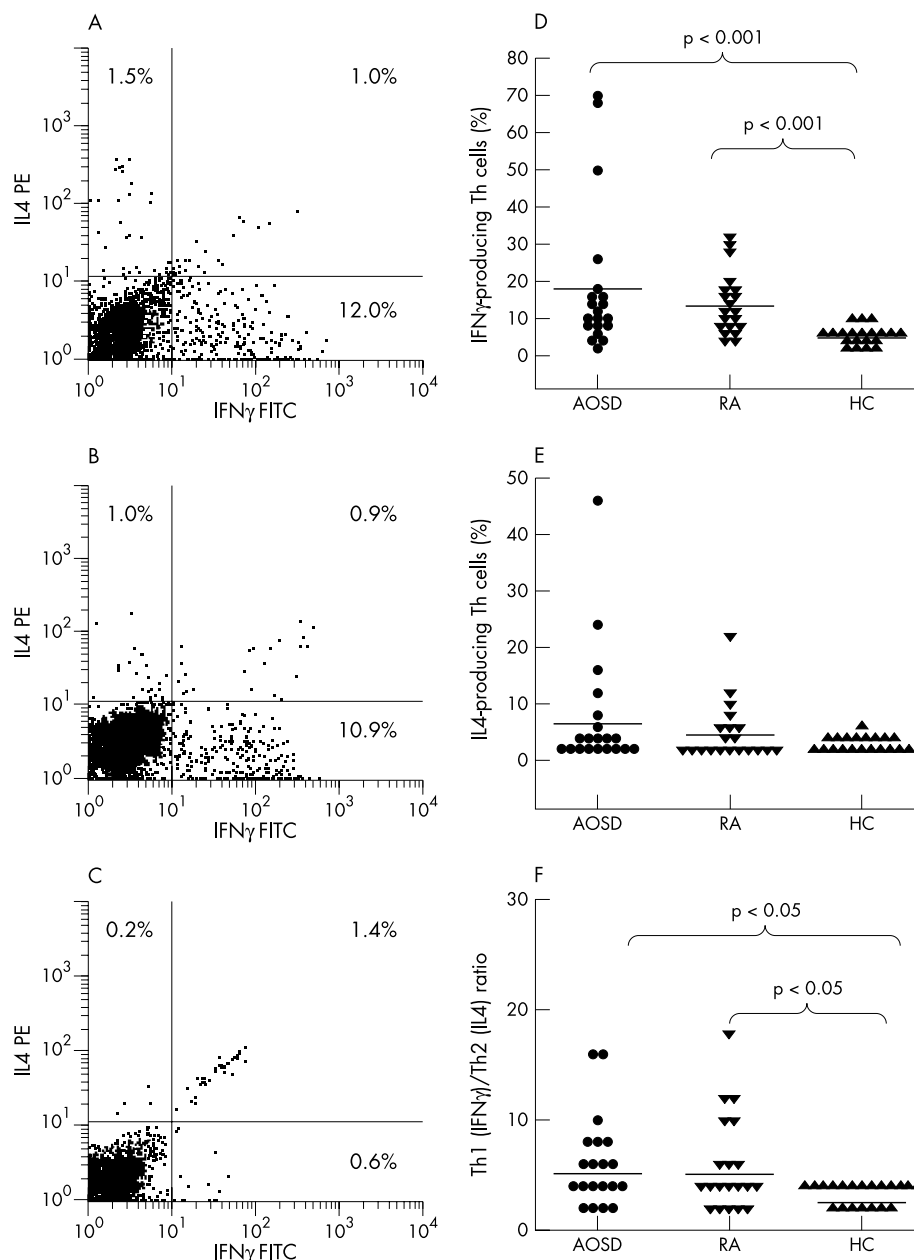
Figure 1 Flow cytometric dot plots of intracellular cytokine production in Th cells obtained from PB of (A) one represented patient with active untreated AOSD; (B) one patient with active RA; and (C) one HC after stimulation with PMA/ionomycin in the presence of brefeldin A. The figure in the quadrants denotes the percentage of cells expressing IFNγ or IL4 in CD4+ T cells. Mean percentages of (D) IFNγ-; (E) IL4-producing Th cells; and (F) the Th1 (IFNγ)/Th2 (IL4) ratio in Th cells obtained from the PB of patients with active untreated AOSD, active RA, and the HC. Horizontal bars indicate means. The p values are provided for comparison of these three groups.

downstream 3'quencher dye. The black hole quencher dyes used are a new class of dark quenchers that have no fluorescence of their own, essentially removing background problems. PCR was performed in a total volume of 50.0 μl containing 100 ng of cDNA, 0.5 μl Taq DNA polymerase (5 U/ μl), 5.0 μl TaqMan probe, 5.0 μl each oligonucleotide primer, 25.0 μl PCR buffer, and 9.5 μl RNase-free water. PCR conditions were incubation for 2 minutes at 50°C, activation of Taq DNA polymerase for 10 minutes at 95°C, and then 40 cycles of 95°C for 15 seconds, followed by 58°C for 1.5 minutes. Each run included external standards as positive controls, water without template as negative controls, and samples without Taq DNA polymerase as controls for fluorescent contaminants. The cDNA concentration in each

sample was then calculated automatically by reference to the standard curve. To standardise cytokine mRNA concentrations, transcript levels of the housekeeping gene β-actin were determined in parallel for each sample.

Determination of serum levels of IL18 and sIL2R

Serum levels of IL18 were determined by an ELISA kit (Bender MedSystems, Austria). The lower limit of detection was 30.0 pg/ml. For statistical analysis, this value was arbitrarily attributed to the samples containing IL18 levels below this detection threshold. Serum sIL2R levels were determined by an ELISA kit (Cellfree; Endogen Inc, MA, USA). The overall intra- and interassay coefficients of variation in this study were 2.9% and 12.5%, respectively.
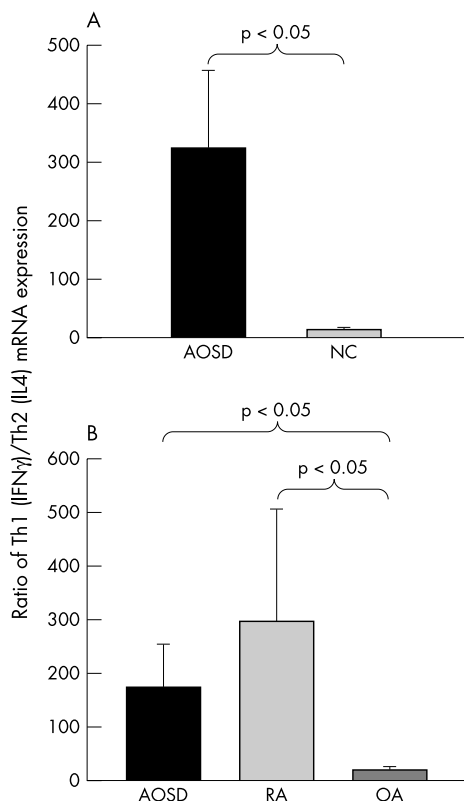
Figure 2 The ratio of Th1 (IFNγ)/Th2 (IL4) mRNA expression in the biopsy skin (A) of evanescent rash from patients with AOSD (n = 8) and from normal controls (NC, n = 4), and in the biopsy synovium (B) of active synovitis from patients with AOSD (n = 6), of active synovitis from patients with RA (n = 6) and OA controls (n = 4) was analysed by the real time TaqMan PCR method. Data are presented as mean (SEM). The p values are provided for comparison of different groups.

Figure 3 Serum levels of IL18 in patients with active untreated AOSD, active RA, and healthy controls (HC). Horizontal bars indicate means. The p values are provided for comparison of the three groups.

Statistical methods

Results are presented as the mean (SD) unless specified otherwise. The non-parametric Kruskal-Wallis test was used for between-group comparison of serum levels of IL18, the percentages of cytokine-producing Th cells in PB, and the Th1/Th2 ratio. Only when this test showed significant differences were the exact p values determined using the Mann-Whitney U test. The ratio of Th1/Th2 cytokine mRNA expression in biopsy specimens between groups was compared by the Kruskal-Wallis test or the Mann-Whitney U test, as appropriate. The correlation coefficient was obtained by the non-parametric Spearman's rank correlation test. For comparison of the percentage of cytokine-producing Th cells and the Th1/Th2 ratio during follow up for patients with AOSD before and after GC and MTX treatment, the Wilcoxon signed rank test was employed. A probability of less than 0.05 was considered significant. Statistical calculations were performed using the Statistical Package for Social Science (SPSS) for Windows, version 10.0.

RESULTS

Clinical characteristics of the patients with AOSD and RA

All 20 patients with active untreated AOSD had daily spiking fevers (⩾39°C) and articular symptoms. Typical evanescent rash and sore throat were present in 17 (85%) patients. Lymphadenopathy and hepatomegaly were noted in seven (35%) and six (30%) patients, respectively. All patients with RA had active disease (de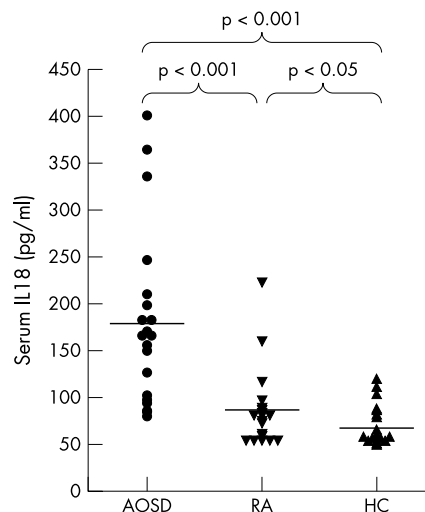fined as six or more swollen and six tender joints). The mean duration of morning stiffness was 13.0 hours (range 5.0–17.0).

Mean percentages of cytokine-producing Th cells and Th1/Th2 ratios in PB of patients with AOSD and RA

Figures 1A–C show representative examples of flow cytometric dot plots of intracellular cytokine production in Th cells obtained from the PB of one patient with active untreated AOSD, one patient with active RA, and one healthy control (HC). The mean percentages of IFNγ-producing Th cells were significantly increased in patients with active untreated AOSD and in patients with active RA relative to the healthy controls (17.94 (20.04) v 4.76 (2.26), p<0.001 and 13.31 (8.24) v 4.76 (2.26), p<0.001, respectively) (fig 1D). No significant differences were found between either of the patient groups and healthy controls in the percentages of IL4-producing Th cells (fig 1E). The Th1 (IFNγ)/Th2 (IL4) ratio was significantly higher in patients with active untreated AOSD than in the healthy controls (5.12 (4.00) v 2.50 (0.96), p<0.05) and was also higher in the patients with active RA (5.05 (4.39) v 2.50 (0.96), p<0.05) (fig 1F). No significant difference in the percentages of cytokine-producing Th cells and the Th1/Th2 ratio was seen between patients with active untreated AOSD and patients with active RA.

Ratio of Th1 (IFNγ)/Th2 (IL4) mRNA expression in biopsy specimens of evanescent rash and synovitis from patients with active untreated AOSD

The ratio of Th1 (IFNγ)/Th2 (IL4) mRNA expression in biopsy specimens of evanescent rash was significantly higher among patients with active untreated AOSD than among normal skin controls (323.7 (379.8) v 12.6 (5.5), p<0.05) (fig 2A). The ratio of Th1/Th2 cytokine mRNA expression in SM was significantly higher in patients with active untreated AOSD than in OA controls (175.0 (198.4) v 19.5 (10.9), p<0.05) and was also higher in patients with active RA than in OA controls (294.4 (517.1) v 19.5 (10.9), p<0.05) (fig 2B). There was no significant difference in the ratio of Th1/Th2 cytokine mRNA expression in SM between patients with AOSD and patients with RA.

Serum IL18 levels of patients with AOSD and RA

The serum levels of IL18 were significantly increased in patients with active untreated AOSD compared with patients with active RA (178.9 (93.3) pg/ml v 85.8 (41.0) pg/ml,
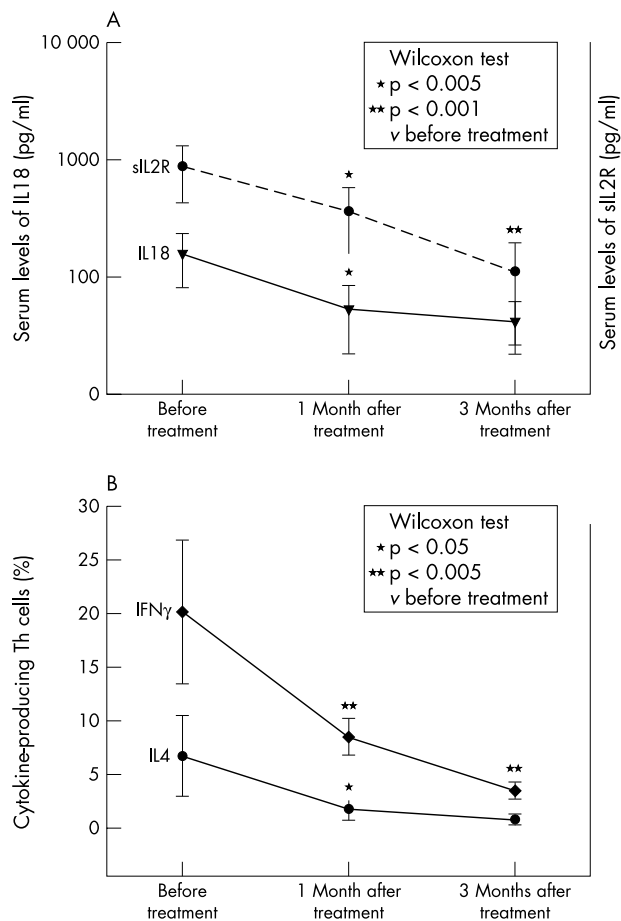
Figure 4 Serial changes in (A) the levels of IL18 and sIL2R in serum, and in (B) the mean percentage of IFNγ-and IL4-producing Th cells in PB of 12 patients with AOSD before treatment and at 1 and 3 months after treatment with GC, NSAIDs, and methotrexate. Data are presented as mean (SEM).

p<0.001) and the healthy controls (178.9 (93.3) pg/ml v 67.3 (21.5) pg/ml, p<0.001) (fig 3). The serum IL18 levels were also significantly increased in patients with active RA in comparison with the healthy controls (p<0.05) (fig 3).

Correlation between the percentages of cytokine-producing Th cells or Th1/Th2 ratio in PB and clinical activity score and serum IL18 levels of patients with AOSD

The percentages of IFNγ-producing Th cells in PB of patients with AOSD was significantly correlated with clinical activity score ($r_s = 0.486$, p<0.05) and serum IL18 level ($r_s = 0.475$, p<0.05). The Th1/Th2 ratio in PB for patients with AOSD was also significantly correlated with clinical activity score ($r_s = 0.554$, p<0.05) and serum IL18 level ($r_s = 0.660$, p<0.005). However, the correlation between the percentage of IL4-producing Th cells and clinical activity score or serum IL18 levels in the patients with AOSD did not reach statistical significance.

Changes in serum level of IL18 and sIL2R, percentage of cytokine-producing helper T cells, and the Th1/Th2 ratio in PB of patients with AOSD after treatment

After 3 months of treatment, complete (n = 15) or partial (n = 5) remissions had been achieved for all patients with AOSD. The serum levels of IL18 and sIL2R declined markedly, paralleling the clinical remission (p<0.005 for both, fig 4A).

To investigate the Th1/Th2 cytokine changes, 12 patients with AOSD were available for examination at an arranged time in the first and the third month after the start of treatment. Three months of treatment with GC and MTX resulted in a rapid and sustained decrease in the mean percentage of IFNγ-producing Th cells, approaching the levels in healthy controls (p<0.005; fig 4B). After 3 months of treatment, however, the percentages of IL4-producing Th cells showed no significant change (fig 4B). The Th1/Th2 ratio in PB decreased after 3 months' treatment (7.47 (7.20) v 3.58 (4.90), p = 0.05).

DISCUSSION

As far as we know this study is the first to demonstrate an imbalance in the Th cell cytokine profile at the single cell level in the PB of patients with active untreated AOSD. To obtain a better reflection of in vivo cytokine patterns than is achievable with mononuclear cells from PB, whole blood was analysed by flow cytometry, with staining of the intracellular cytokines and extracellular surface markers. To increase the signal to noise ratio, we used direct conjugation of monoclonal anticytokine antibodies, as used in a previous study.[25] When short term cell stimulation combined with blockade of cytoplasmic transport of cytokine was used, a highly significant correlation was found between the percentage of cytokine-producing cells and the amount of that specific cytokine in the cell culture supernatant.[26] Furthermore, the approach in this study is better than an ELISA of supernatant cytokines because the investigation of cytokine profiles is not influenced by the soluble cytokine receptors or inhibitors in serum or plasma. We showed that the mean percentages of IFNγ-producing Th cells were significantly higher for patients with active untreated AOSD than for healthy controls (p<0.001, fig 1D). However, there was no significant difference in the percentage of IL4-producing Th cells between patients with active untreated AOSD and healthy controls (fig 1E). Our data are consistent with previous studies that showed no increase in serum IL4 levels in systemic AOSD[8] and decreased IL4-producing Th cells in PB from subjects with juvenile onset Still's disease.[27] The Th1 (IFNγ)/Th2 (IL4) ratio in PB was significantly higher in our patients with active untreated AOSD than in controls (p<0.05, fig 1F). In addition, the percentages of IFNγ-producing Th cells and the Th1/Th2 ratio correlated significantly with clinical activity score in the patients with AOSD ($r_s = 0.486$ and $r_s = 0.554$ respectively, p<0.05 for both). Our results were comparable with previous reports that showed raised serum Th1-cytokine levels in patients with AOSD[5 8] and a marked Th1 response in synovial fluid mononuclear cells of JRA.[28] Serum levels of IL12, which has been shown to prime the selective expansion of Th1 cells, were raised in patients with active JRA including the group with systemic disease.[29 30] The predominance of Th1 cytokine may also explain the absence of antinuclear antibodies and rheumatoid factors in patients with AOSD. However, it has recently been demonstrated using an ELISA that serum IL4 levels are increased in patients with chronic articular AOSD during the active phase,[8] that a mixed Th1/Th2 cytokine response predominates in patients with juvenile onset Still's disease,[31] and that a decrease in Th1 cytokine production of PB mononuclear cells from patients with RA correlates with an increase in activity of disease.[32] This disparity in Th1/Th2 balance may be due to differences in population characteristics and the detection method used.

IL18, a recently described member of the IL1 cytokine family, promotes a Th1 immune response and stimulates the synthesis of IFNγ in T cells and natural killer cells in synergy with IL12.[17 33] Serum IL18 levels are increased in several inflammatory diseases, including AOSD and RA, during the

active stage.[6 8 34 35] The notion of a role for IL18 in RA pathogenesis is supported by a recent report, which demonstrated that IL18 is present at a significant level in the inflammatory rheumatoid synovium, where it induces a Th1 response.[35] We demonstrated significantly higher levels of serum IL18 for patients with active untreated AOSD and active RA than for healthy controls, consistent with the results of previous studies.[6 8] In addition, the serum IL18 levels of our patients with AOSD correlated significantly with the percentages of IFNγ-producing Th cells ($r_s = 0.475$, p<0.05) and the Th1/Th2 ratio ($r_s = 0.660$, p<0.005). One recent study indicated that IL18 may potentiate IL12-induced IFNγ production by T cell infiltrates in RA synovium.[35] Kawashima et al demonstrated that serum samples from patients with active AOSD could induce IFNγ secretion from human myelomonocytic KG-1 cells, which was largely abolished by neutralising anti-IL18 antibody.[6] Our results suggest that IL18 overproduction may be related to the Th1 polarisation of patients with AOSD. Interestingly, serum levels of IL18 were markedly higher in our patients with active untreated AOSD than in patients with active RA (fig 3). The significant difference in serum IL18 levels between AOSD and RA groups may reflect disparity in some aspect of disease pathogenesis. However, bias may have been introduced as a result of MTX treatment in patients with RA before this investigation.

To verify the Th1 predominance in PB found by flow cytometry, RQ-PCR for Th1 and Th2 cytokine mRNA expression was performed in biopsy specimens of evanescent rash and synovitis from patients with active untreated AOSD. We have demonstrated for the first time that the ratio of Th1/Th2 cytokine mRNA expression is significantly higher in the biopsy specimens of evanescent rash and synovitis from patients with AOSD than in the specimens of normal skin and in OA synovium, supporting a predominantly Th1 pattern of cytokine mRNA expression in the pathological tissues of patients with AOSD. In this study the cytokine pattern of mRNA expression on synovium of patients with AOSD was similar to that of patients with RA and JRA, in which a predominance of Th1 cytokine mRNA expression has been demonstrated on synovium.[16 36] The RQ-PCR method used in this study has been shown to be sensitive and reliable for quantifying mRNA expression even with a small amount of tissue.[37] Moreover, previous studies demonstrated that the pattern of cytokine gene expression in synovium parallels the profile of cytokines released in synovial explant supernatant,[38] and the levels of cytokine gene expression in PB mononuclear cells correlate well with the amount of the corresponding cytokine secreted by these cells.[39] The Th derived cytokine pattern of mRNA expression in the pathological tissues was in agreement with the polarisation of Th cells in the PB of our patients with AOSD, suggesting that a predominance of Th1 cytokine may participate in the pathogenesis of this disease. However, the numbers of patients studied in this RQ-PCR investigation were small and therefore these data have to be confirmed in a larger study.

During a longitudinal follow up of Th1 and Th2 cytokine changes in PB of 12 patients with AOSD, we found that the serum levels of IL18 and sIL2R, and the mean percentage of IFNγ-producing Th cells declined significantly, paralleling the clinical remission after 3 months of GC and MTX treatment (figs 4A and B). In contrast, the mean percentage of IL4-producing Th cells was not significantly decreased after 3 months of treatment. Although there was no statistical significance, a decrease in the Th1/Th2 ratio was observed among these patients (p = 0.05). The change in serum levels of sIL2R and IL18 in this study was compatible with previous reports showing that levels of sIL2R and IL18 correlate with

disease activity in patients with chronic articular AOSD.[8] Schulze-Koops et al showed that the Th1 cytokine production by mitogen-stimulated peripheral blood (PB) T cells and the levels of Th1 cytokine mRNA expression in PB mononuclear cells from patients with RA were significantly reduced after treatment with anti-CD4 antibody.[39] In contrast, no consistent changes could be observed for Th2 cytokine (IL4). Possibly, therefore, a shift from a Th1 dominated immune response towards a Th2 response, paralleled the clinical improvement.[40] Our results were also similar to those of a recent study that showed higher levels of Th1 cytokine (IFNγ) in supernatants of PB lymphocytes from patients with steroid sensitive nephrotic syndrome during relapse compared with those in remission.[41] Further, Th derived cytokine profiles may be influenced by the treatment employed. Modulation of the cytokine network by GC, which suppressed Th1 immune response by both inhibiting IL12 secretion and Stat4 mediated IL12 signalling,[42] and by MTX, which decreased the expression of IFNγ genes of PB mononuclear cells obtained from patients with RA and healthy controls,[43] might explain the change of Th1/Th2 during the treatment of our patients with AOSD. The lack of a significant shift in Th1/Th2 pattern after the 3 months' treatment may be explained by short duration of follow up, relatively small sample population, or by a combination of both.

In conclusion, our results suggest that there was a predominance of Th1 cytokine in both PB and pathological tissues of patients with active untreated AOSD. Determination of the Th1/Th2 imbalance not only improves understanding of immunopathogenesis of AOSD but it also offers a foundation for the development of a new therapeutic strategy.

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