

## Levels of soluble CD27 in sera and synovial fluid and its expression on memory T cells in patients with juvenile idiopathic arthritides

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### ABSTRACT

**Objective.** CD27 is a member of tumour necrosis factor receptor family. Its expression is predominantly confined to mature lymphocytes and is strongly enhanced after cell activation. Shedding of the CD27 from the surface of activated cells is related to their effector phase. The aim of the present study was to evaluate the levels of soluble CD27 in sera and synovial fluids, together with its expression on circulating and synovial fluid (SF) memory T cells, in children with JIA.

**Methods.** Sera from 40 patients with active JIA were studied for soluble CD27. Paired SF samples were available in 20 patients. Sera from 12 age-matched patients affected with various acute infectious diseases and 12 age-matched healthy subjects were used as controls. In 8 JIA patients freshly isolated circulating and SF lymphocytes were stained for CD27 in CD4+CD45RO+ T cell subpopulation and analyzed by cytometry.

**Results.** Soluble CD27 serum levels were significantly higher in patients with polyarticular JIA and acute systemic infectious diseases than in patients with active oligoarticular or healthy controls. Both polyarticular and oligoarticular JIA patients showed increased levels of soluble CD27 in SF when compared with paired serum samples ( $p=0.01$ ). In all the patients tested a significant enrichment of CD27+ T cells was seen in the SF (median 39.5%, range 18-56%) when compared to paired CD4+CD45RO+ peripheral lymphocytes (median 19.5%, range 5-43%;  $p=0.01$ ).

**Conclusions.** A clear enrichment of CD4+ memory SF T cells with a CD27-phenotype is observed when compared to correspondent circulating T lymphocytes. This issue is conceivably related to re-activation and recruitment of memory T cells to the site of inflammation, and to the subsequent expansion of a subpopulation of "effector" memory T cells.

### Introduction

T lymphocytes are thought to play a pivotal role in the initiation and perpetuation of chronic synovitis in the hu-

man inflammatory arthritides, including juvenile idiopathic arthritides (JIA) (1-3).

CD27 is a member of the tumour necrosis factor receptor (TNFR) family. Its expression is predominantly confined to mature lymphocytes and is strongly enhanced after cell activation (4). This molecule has been shown to stimulate T cell proliferation and B cell differentiation through the interaction with its specific ligand, namely CD70. Persistent antigenic stimulation induces the proteolytic cleavage of the soluble 32 kDa form of CD27 from the cell surface (4). According to previous studies such process correlates with the degree of T cell activation, both at the systemic and the local level. In fact, increased levels of soluble CD27 have been found both in sera of renal transplant recipients (5) and in cerebrospinal fluid of patients affected with multiple sclerosis (6).

According to the model proposed by Van Lier *et al.*, naïve T cells constitutively express CD27, which is up-regulated following antigen presentation and T cell activation. Interactions between the T cell co-stimulatory molecule CD28 and its ligands (CD80/CD86) on antigen presenting cells (APC) is followed by the release of pro-inflammatory cytokine from APC, which eventually results to the expression of CD70 on activated T cells. The CD27-CD70 interaction will further support clonal expansion of antigen specific T cells, the majority of which will subsequently differentiate into effector cells, shedding the CD27 from their surface in fluid phase.

Conversely, a subset of antigen-specific T cells that does not differentiate into effector cells circulate in the blood as CD27+ "resting" memory T cells, ready to undergo to re-activation upon secondary exposure to antigen ("early" memory) and subsequent differentiation into "effector" CD27- memory cells (7).

The concept the CD4+CD45RO+CD27- cell subset associates with the effector phase of memory T cells differentiation is supported by the following observations: i) it is a potent inducer of B cell differentiation (and Ig production) (8);

ii) it is enriched for cells producing IL-4 or IFN- $\gamma$  (9, 10); iii) it expresses organ-specific homing receptors, such as CLA and the  $E_{7}$  integrin (11, 12). Soluble CD27 has been found to be significantly increased in the synovial fluid of patients affected with rheumatoid arthritis (RA) in comparison to patients with osteoarthritis (13). Moreover, CD27 has been shown to be expressed in different proportion and distribution, particularly in the subset of memory T cells (CD4+CD45RO+) both in the synovial fluid and in the inflamed synovial tissue from RA patients (13, 14).

Aim of the present study was to evaluate the levels of soluble CD27 in sera and synovial fluids, together with its expression on circulating and synovial fluid memory T cells, from children affected with JIA.

### Patients and methods

#### *Soluble CD27 serum and SF concentrations*

Sera from 40 consecutive patients affected with active JIA according to Durban criteria were studied between (15). Fifteen patients displayed a polyarticular course of the disease (5 with systemic onset, 4 with RF-negative polyarticular onset, 1 with RF-positive polyarticular onset and 5 with extended oligoarticular course). Twenty-five had an oligoarticular course. A serum sample was collected with permission at each control visit or at the moment of hospitalization for a disease flare-up and stored at  $-80^{\circ}$  immediately after centrifugation.

Paired synovial fluid (SF) samples were collected after therapeutic arthrocentesis in 20 patients (7 with polyarticular and 13 with oligoarticular course).

A number of clinical (no. of active joints, no. of joints with limited range of motion and physician global assessment of overall disease activity) and laboratory parameters (erythrocyte sedimentation rate, ESR; C-reactive protein, CRP; white blood cell, WBC and platelet, PLT, counts; hemoglobin serum concentration, Hb) of disease activity were recorded, together with the ongoing treatment, at the time of the study (Table I).

Disease activity was defined by the presence of active arthritis (swelling or, if swelling was absent, limitation of motion with tenderness) at least in one joint at the moment of clinical examination and a physician global estimate of disease activity (measured on a 0-10 cm visual analog scale) higher than 1 (16,17).

Sera from 12 previously healthy age-matched patients affected with various acute febrile infectious conditions (4 with bacterial pneumonia, 3 with streptococcal pharyngitis, 2 with pharyngitis, 2 with acute mononucleosis, 1 with Salmonella gastroenteritis) were used as positive, "inflammatory" controls.

Twelve age-matched healthy subjects attending at our clinic for routine pre-operative (orchidopexy, phimosis correction ...) examination were used as negative controls after informed consent of the parents. History of inflammatory or infectious disorders in the 4

weeks before the examination, together with clinical or laboratory (i.e. elevated ESR or CRP) signs of inflammation at the moment of the study, were considered as criteria of exclusion.

Soluble CD27 in sera and SF was determined using a commercial ELISA kit (CLB, The Netherlands), according to the manufacturer's instructions.

Expression of CD27 on peripheral and SF memory T cells. Peripheral blood (PB) and synovial fluid (SF) mononuclear cells were isolated from heparinized blood and synovial samples from 8 JIA patients (2 poly and 6 oligo) by Ficoll (Biochrom KG-Germany) density gradient centrifugation. PB mononuclear cells were also isolated from two age-matched healthy controls. Cells were washed and a three-color staining was performed with CD45RO-tricolor mAb (Caltag-Burlingame-Ca), CD4-FITC or -PE mAb (BD Biosciences-Mountain View-Ca) and CD27-PE or -FITC mAb (PharMingen, San Diego, CA). Cells were incubated with saturating amounts of mAbs for 30' at  $4^{\circ}\text{C}$ , then cells were washed in PBS (Biowhittaker-Walkersville-USA) with 1% FCS (Biochrom) and analyzed by flow cytometry (FACScan-BD Biosciences) gating on the CD4+CD45RO+ T cell subset. PE-, FITC- and tricolor-conjugated mouse Ig control mAbs (Caltag) were used as negative controls. CellQuest software (BD Biosciences) was used for analyses.

#### *Statistical analysis*

Serum levels of soluble CD27 were compared among four subgroups of pa-

**Table I.** Clinical and laboratory features (mean, range) of JIA patients at the moment of the study.

	Age	Disease duration (yrs.)	No. of joints active/limited range of motion	Physician's global index	Erythrocyte sedimentation rate	Treatment
Polyarticular course (15 pts)	7.2 (3.3 – 16.1)	3.7 (0.5 – 11.2)	7.2 /12.2 (1-16)/(1-29)	8.5 (4.6 – 10)	76 (23-128)	NSAID, MTX (8 pts.) NSAID, CS, MTX (3 pts.) NSAID, CS (2 pts.) NSAID alone (2 pts.)
Oligoarticular course (25 pts)	8.5 (3 – 17.9)	2.4 (0.3 – 14)	1.3 /1.5 (1-3)/(1-3)	6.8 (3 – 9.1)	28.5 (7 – 76)	NSAID alone (12 pts.) NSAID, CS (5 pts.) NSAID, MTX (3 pts.) Nihil (5 pts.)

NSAID: non steroidal anti-inflammatory drugs; MTX: methotrexate; CS: corticosteroids.

tients (active polyarticular JIA patients, active oligoarticular JIA patients, acute febrile infectious disease, healthy controls) using the non-parametrical U Mann-Whitney test. Correlations among all the variables considered were analyzed using the non-parametric Spearman rank test. Differences in soluble CD27 concentrations between paired serum and SF and difference of the percentage of CD27 positive cells in the CD4+CD45RO+ subpopulation between peripheral and SF lymphocytes were determined with the Wilcoxon rank test.

## Results

### Soluble CD27 serum and SF concentrations

Soluble CD27 serum levels were significantly higher both in patients with polyarticular JIA (median 415.7 U/mL, range 206-864) or in acute systemic infectious diseases (437 U/mL, range 220-750) than in patients with active oligoarticular JIA (247 U/mL, range 100-800;  $p=0.05$  and  $p=0.004$ , respectively) or healthy controls (198 U/mL, range 88-383 U/mL;  $p=0.04$  and  $p=0.004$ , respectively) (Fig. 1, panel A). No statistical differences were noted between patients with active polyarticular JIA and children with infectious diseases, and between oligoarticular JIA patients and healthy controls.

In polyarticular JIA, sCD27 serum concentrations displayed a significant correlation with some clinical (physician global estimate of disease activity  $r=0.85$ ,  $p=0.001$ ) and laboratory (ESR:  $r=0.61$ ,  $p=0.03$ ; CRP:  $0.63$ ,  $p=0.02$ ) parameters of disease activity. Conversely, no statistically significant correlations were found in the active oligoarticular subset.

Both polyarticular and oligoarticular JIA patients showed a significantly increased levels of sCD27 in SF (median: 1013 U/mL, range 323-2580 and 1318 U/mL, range 117-2410; respectively) when compared with paired serum samples ( $p=0.01$ ).

### Expression of CD27 on peripheral blood and SF memory T cells

As expected, an enrichment of CD4+CD45RO+ memory T cells was found in the SF of patients with JIA (median 34%, range 22-50%) when compared to paired peripheral blood mononuclear cells (median 8%, range 4-14%,  $p=0.01$ ). Expression of CD27 was detected both on peripheral and SF memory (CD4+CD45RO+) T cells. The CD27+ subset was the most represented phenotype, both in peripheral blood (median 80.5%, range 56-95%) and in SF (median 60.5%, range 45-82%) memory T cells (Fig. 1, panel B). However, in all the patients tested a significant

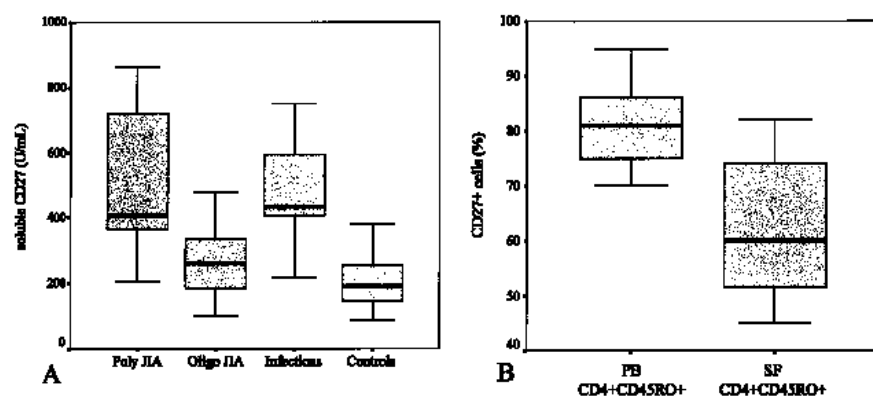
enrichment of CD27+ T cells was seen in the SF (median 39.5%, range 18-56%) when compared to paired CD4+CD45RO+ peripheral lymphocytes (median 19.5%, range 5-43%;  $p=0.01$ ) (Fig. 2). No difference was found in the expression of CD27 on CD4+CD45RO+ circulating T cells among JIA patients and age-matched healthy controls (81% in control 1, 86% in control 2).

## Discussion

Activated T cells are thought to play a crucial role in the pathogenesis of JIA, through their role in the regulation of the inflammatory response after antigen presentation. In the present study, CD27 was used both as a marker of activation and functional characterization of T cells during the active phase of the disease. In particular, according to some Authors, the irreversible shedding of the CD27 from the cellular surface is considered as a reliable marker of their effector phase for recently activated naïve and memory T cells (7, 8, 18).

In the present study, similarly high levels of soluble CD27 were seen both in the JIA patients with a polyarticular course and in the subjects with acute systemic infections. Conversely, no significant difference was observed between patients with a predominantly local inflammation (oligoarticular JIA) and healthy controls. Thus, it is conceivable that serum concentrations of soluble CD27 could be aspecifically related to the degree of systemic inflammation (7). Even if the JIA subtypes with a polyarticular course could not be properly considered as systemic diseases, in these conditions the acute phase reactants are often related to the degree of disease activity (19). In this line, the correlation of soluble CD27 serum levels with some disease activity parameters in the polyarticular patients may be aspecifically related to the activation of circulating naïve and memory T cells (7).

Thus, in order to better elucidate the role of CD27 as a specific marker of T cell differentiation at the site of tissue inflammation, we have investigated the expression of CD27 on synovial fluid memory CD4+ T cells, that represent



**Fig. 1. Panel A.** Serum levels of soluble CD27 in active polyarticular and oligoarticular JIA patients, patients with an acute systemic infection and healthy controls. Soluble CD27 serum levels were significantly higher both in patients with polyarticular JIA or in acute systemic infectious diseases than in patients with active oligoarticular JIA ( $p=0.05$  and  $p=0.004$ , respectively) or healthy controls ( $p=0.04$  and  $p=0.004$ , respectively). **Panel B.** Expression of CD27 on CD4+CD45RO+ synovial fluid (SF) lymphocytes in comparison with paired peripheral blood (PB) lymphocytes.

For both figures: bold horizontal lines represent median values. Boxes contain the 50% of values falling between the 25th and 75th percentiles, whiskers lines that extend from the boxes represent the highest and lowest values for each subgroups.

the main T cell subpopulation found in the synovial tissue in the idiopathic chronic arthritides (1).

Even on a relative small number of patients, a clear-cut enrichment of CD4+CD45RO+CD27- T cells was observed in SF when compared to correspondent circulating T lymphocytes. In this line, the high concentrations of soluble CD27 found in SF due to an increased shedding from the cells surface may represent a marker of local activation of memory T cells at that level (7).

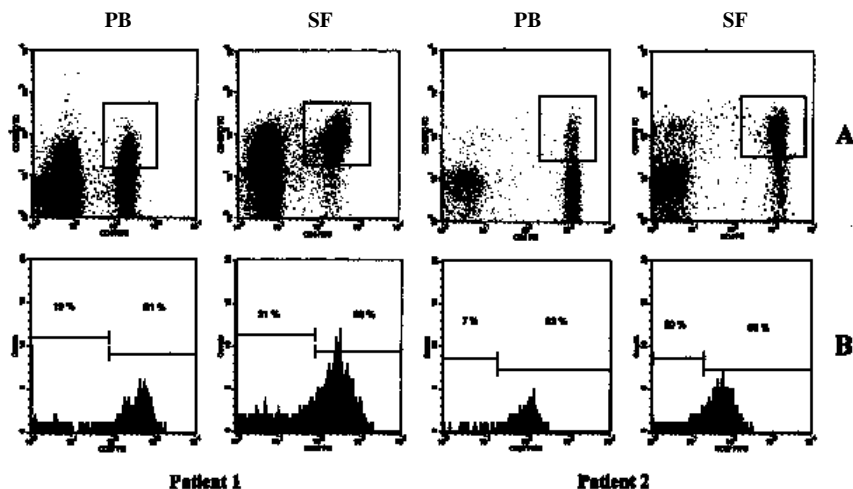
This issue is in line with previous studies on adult RA (13, 14) and is conceivably related to re-activation and recruitment of memory T cells to the site of inflammation, and to the subsequent expansion of a subpopulation of "effector" memory T cells (18).

Notably, the immunohistochemical characterization of rheumatoid synovial tissue in adult RA has shown a prevalent localization of CD4+CD45RO+CD27+ T cells in the perivascular lymphocytic aggregates, with a relative increase of the CD4+CD45RO+CD27- T cells in diffuse lymphocytic infiltrates. Thus, it is conceivable that recently re-activated CD4+CD45RO+CD27+ are recruited from peripheral blood into the synovium to undergo further cycles of activation and differentiation to CD4+CD45RO+CD27- T cells, followed by migration in the context of synovial tissue, where they are thought to exert their effector functions (14).

A further analysis of the functional properties of CD27+ and CD27- memory T cells in JIA is currently ongoing in our laboratory.

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**Fig. 2.** Three-color flow cytometric analysis of paired peripheral blood (PB) and synovial fluid (SF) lymphocytes from two representative JIA patients with oligoarticular (pt. 1) and polyarticular (pt. 2) course. Cells were stained simultaneously with CD45RO-tricolor mAb in combination with CD4-FITC and CD27-PE (pt. 1), or CD4-PE and CD27-FITC (pt. 2). **Panel A.** The dot plots show the expression of CD45RO and CD4 by PB and SF lymphocytes; gates were set on double positive CD45RO+, CD4+ cells. **Panel B.** The expression of CD27 by the gated CD45RO+, CD4+ cells shown in A was analysed. Histograms indicate the percentage of CD27+ (right bars) and CD27- (left bars) cells in the gated populations. The percentage of positive and negative cells for the different Ag was calculated on the basis of appropriate isotype-matched control mAbs.