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Synovial dendritic cells in juvenile idiopathic arthritis (JIA) express receptor activator of NF- κ B (RANK)

H. Varsani, A. Patel, Y. van Kooyk², P. Woo¹ and L. R. Wedderburn

Objectives. To analyse the expression of receptor activator of NF- κ B (RANK) and RANK ligand (RANKL) in the joints of children with juvenile idiopathic arthritis (JIA), to characterize the phenotype of RANK⁺ cells and to test the hypothesis that some RANK⁺ cells are of the dendritic type.

Methods. Paired samples of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC) from children with oligoarticular ($n=14$) or polyarticular ($n=4$) JIA and PBMC from 10 control subjects were studied for expression of RANK, RANKL and dendritic cell-specific ICAM (intercellular adhesion molecule)-grabbing non-integrin (DC-SIGN) by the reverse transcriptase–polymerase chain reaction and three-colour flow cytometry. Expression of DC-SIGN and RANK was followed after 1 week of culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4).

Results. mRNA for RANK was detected in both adherent cells and T cells from PBMC and SFMC of patients with JIA and in control PBMC, while mRNA for RANKL was detectable in the T-cell fraction from JIA patients but not in that from controls. By flow cytometry, a large number of RANK⁺ cells were detected in the joint; these cells had the phenotype HLA-DR^{hi}CD86^{hi} CD11c⁺ and expressed low levels of DC-SIGN.

Conclusions. There is increased expression of RANKL and RANK in the juvenile arthritic joint. RANK is expressed on a population of cells with features of dendritic cells. RANK/RANKL interactions may contribute to the survival of inflammatory cells within the joint, as well as to erosions and osteoporosis in juvenile arthritis.

Juvenile idiopathic arthritis (JIA) is characterized by a persistent inflammatory infiltrate within the synovial layer of the joint. T cells within the hypertrophied synovium are activated, oligoclonal, and have a bias towards a T helper 1 (Th1) phenotype in oligoarticular and polyarticular JIA [1–5]. It is unclear what causes clonal inflammatory lymphocytes to persist over long periods

within the joint [6]. One mechanism may be through signals received from professional antigen-presenting cells such as dendritic cells (DC). This interaction may also be two-way (from T cell to DC), through ligand pairs such as CD40L/CD40 [7]. Two recently identified members of the tumour necrosis factor (TNF) family, the receptor activator of NF- κ B (RANK) and RANK

Rheumatology Unit, Institute of Child Health, University College London and Great Ormond Street Hospital for Children NHS Trust, ¹Department of Molecular Pathology and Immunology, University College London, London, UK and ²Department of Immunology, Free University Medical Centre, Amsterdam, The Netherlands.

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Correspondence to: L. R. Wedderburn, Rheumatology Unit, Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, UK. E-mail: l.wedderburn@ich.ucl.ac.uk

ligand (RANKL), also known as TRANCE or OPGL [8], have important roles in the interaction between T cells and DC and that between osteoclasts and osteoblasts [9]. RANKL is expressed by activated T cells [10], in particular by Th1 cells [11, 12]. Stimulation of DC through RANK leads to increased DC survival and production of inflammatory cytokines [11, 13, 14]. RANK is also expressed by osteoclasts, where ligation by RANKL leads to activation and maturation, with a consequent increase in bone resorption [15, 16]. In animal models, blocking this interaction using the soluble decoy receptor osteoprotegerin can prevent bone and cartilage destruction [17], while RANKL knockout mice are protected from the erosions associated with arthritis [18]. Expression of RANK and RANKL has been demonstrated in cells and tissues from rheumatoid arthritis (RA) patients [19–21] but the precise identification of the RANK⁺ myeloid cells in arthritis is unclear. To our knowledge there are no previously published studies of RANK or RANKL expression in JIA.

Children with JIA have local destruction of cartilage and, in some cases, bone erosions. They may develop osteopenia or frank osteoporosis, which can occur early in disease and are more severe than would be expected to occur due to steroid use, since the latter is now much reduced in children [22–25]. As JIA occurs during the years of growth, when it is critical to establish peak bone mass, diminished bone density has implications long into adult life, even after disease activity may be reduced [26, 27]. A molecular understanding of the mechanisms which contribute to osteoporosis in JIA is therefore important. In order to consider the use of RANK- or RANKL-blocking therapies in children, the expression of this ligand pair must initially be characterized in JIA. Here we present the first report of the expression of RANK and RANKL in JIA. We show that both are over-expressed in the synovium and that RANK⁺ cells express the DC-specific adhesion receptor DC-specific ICAM (intercellular adhesion molecule)-grabbing non-integrin (DC-SIGN) [28, 29]. We suggest that interactions between RANK and RANKL, expressed both on immune and bone cells, play a role in the pathogenesis of JIA by contributing to the survival of inflammatory cells and increasing osteoclast activity.

Materials and methods

Patients and samples

Samples from 18 children with JIA (14 oligoarticular and four polyarticular onset), five healthy adults and five healthy control children were obtained with full informed consent. The study was approved by the Research Ethics Committee of the Great Ormond Street Hospital Trust. All patients fulfilled the criteria for JIA according to the International League of Associations for Rheumatology (ILAR) classification [30]. Paired samples of peripheral blood (PB) and synovial fluid (SF) were obtained at the time of clinically indicated arthrocentesis. PB mononuclear cells (PBMC) were isolated by standard Ficoll–Hypaque

density centrifugation. For the preparation of synovial fluid mononuclear cells (SFMC), samples were treated with hyaluronidase (Sigma, Poole, UK; 10 U/ml for 30 min at 37°C) before density gradient isolation. In some experiments cells adherent to plastic were enriched by incubation at 37°C for 60 min. T cells were purified by negative selection (i.e. by removal of all non-T cells from the PBMC) in order to prevent any stimulation which might have occurred as a result of the use of positive selection methods, through binding of anti-CD3 antibodies, and which might artificially have altered RANKL expression. Negative selection was performed using antibodies to proteins expressed by (i) cells of the monocyte lineage, i.e. anti-CD14 (UCHM1) (generously donated by Professor P. Beverley) and anti-CD13 (Pharmingen, San Diego, CA, USA), (ii) B cells, i.e. anti-CD19 (BU12) (generously donated by Professor P. Beverley) and (iii) NK cells, i.e. anti-CD16 (Sigma). Cells that were bound by these antibodies were then removed by incubation with anti-mouse IgG-coated magnetic beads (Miltenyi Biotech, Bisley, UK). The purity of the resulting T cells was confirmed by flow cytometry and was routinely >96%.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from 2×10^6 T cells and approximately 2×10^6 adherent cells using RNeasy Lysis Buffer (Qiagen, Crawley, UK) according to the manufacturer's instructions. Five micrograms of total RNA was used in first-strand cDNA using MMLV reverse transcriptase, Superscript II (Gibco, Paisley, UK) and oligo-dT (Boehringer Mannheim, Sussex, UK). Each PCR reaction was performed using 1/60 of the cDNA prepared. The PCR primers were as follows: RANK, 5'-TGACTATCCTGTTCTGTGGGG, 3'-GCGGGTAAGGTCACATACTGA; RANKL, 5'-CTATTTTCAGAGCGCAGATGGAT, 3'-TATGAGAACTTGGGATTTTGTATGC; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5'-TGAAGGT-CGGAGTCA ACGGATTTGGT, 3'-CATGTGGGCCATGAGGCTCACCAC. Twenty per cent of the PCR reaction was run on a 1.5% agarose/TBE gel and the products were visualized by staining with ethidium bromide.

Flow cytometry

Three-colour flow cytometry was performed by standard methods. Monoclonal antibody to human DC-SIGN (clone AZN-D1) was as described previously [29]. Other surface proteins studied were: CD14, expressed on monocytes and macrophages but down-regulated on mature DC; CD13, expressed on all cells of the myeloid lineage; MHC class II protein HLA-DR, up-regulated on maturing DC; CD86, a costimulatory molecule expressed on B cells and macrophages, up-regulated on mature DC; CD11c, the α subunit of the $\alpha_x\beta_2$ integrin and part of a receptor for complement (CR4), expressed on DC; and CD51 (a marker of osteoclasts) and the RANK molecule [8]. The reagents used to detect these were as follows: anti-CD14 fluorescein isothiocyanate (FITC) anti-CD11c-FITC; anti-HLA-DR-FITC; anti-mouse-IgG-FITC; anti-mouse-IgG-biotin; avidin-quantum red (QR/cy) (Sigma); anti-CD86-FITC (Serotech, Oxford, UK); anti-RANK-biotin (R & D Systems, Abingdon, UK); anti-CD13-phycoerythrin (PE); and anti-CD51 (α subunit of vitronectin receptor) (Pharmingen, San Diego, CA). Data were collected on a FACScan analyser (Becton Dickinson, Mountain View, CA, USA). Approximately 20 000 events were collected per condition and data were analysed using Cellquest (Becton Dickinson) software. For analysis of the myeloid cell

population, cells were first gated on the myeloid population by their scatter properties.

Culture of immature dendritic cells

PBMC or SFMC were incubated at $1-2 \times 10^6$ cells/ml for 2 h at 37°C in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin 100 µg/ml, 2 mM glutamine and 5% human AB serum (all from Sigma). Non-adherent cells were removed and each well was washed before culture in fresh medium supplemented with 50 ng/ml interleukin (IL)-4 (R & D Systems) and 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (R & D Systems) for 7 days.

Statistics

Data were processed using the SPSS for Windows statistical package, version 8.1 (SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to determine the normality of data. Student's paired *t*-test was used to compare the expression of the RANK protein on PBMC and SFMC.

Results

Detection of RANK and RANKL mRNA in JIA

RT-PCR analysis was performed on equal numbers of separated T cells and adherent cells from both the PB and SF compartments of children with JIA and PBMC of healthy controls. RANK mRNA was detectable in all samples. RT-PCR data from five representative patients and three controls are shown in Fig. 1. RANKL mRNA was detectable at low levels in the T cells from PBMC and SF T cells of JIA patients, but not detectable in the adherent cells from either compartment (Fig. 1). RANKL mRNA was not detected in either T cells or adherent cells from any of the normal controls (Fig. 1).

High expression of RANK protein on myeloid cells within the joint

Analysis by flow cytometry of RANK expression on SFMC and PBMC from 11 JIA patients revealed that a large subset of myeloid cells within the joint expressed

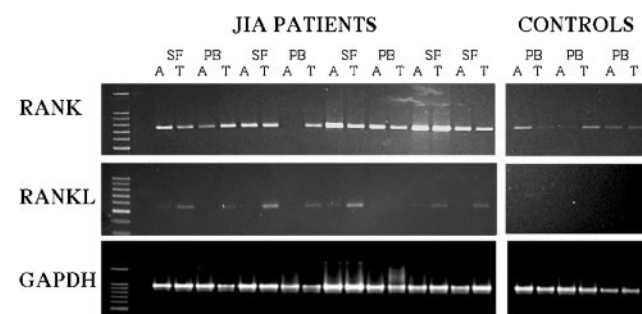


FIG. 1. RT-PCR analysis of the expression of RANK, RANKL and GAPDH mRNA in cells obtained from SF and PB of patients with JIA and PB of healthy controls. For two of the patients illustrated, only SFMC were analysed. First-strand cDNA was prepared from 2×10^6 plastic-adherent cells (A) or T cells (T) from each sample of mononuclear cells, and used to amplify mRNA for the three genes as shown. Twenty per cent of the resultant PCR product was analysed on a 1.5% agarose gel and visualized with ethidium bromide.

high levels of RANK protein while very few RANK⁺ cells were detected in peripheral blood (Fig. 2A). Myeloid cells within the synovial fraction could be divided into three populations by expression of CD14: CD14^{hi}, CD14^{dim} and CD14^{neg}. The majority of the CD14^{dim} cells were also RANK⁺. RANK⁺ cells were CD13⁺CD14^{dim} in each SFMC sample analysed. This was interesting, given that maturing dendritic cells have the phenotype CD13⁺CD14^{dim}. Mean expression of RANK on SFMC myeloid cells was significantly higher than that on PBMC myeloid cells in the set of JIA samples ($n = 11$; mean \pm S.D.: SFMC, 44.8 ± 23.8 ; PBMC, 9.0 ± 9.2 ; $P < 0.001$) (Fig. 2B). There was no significant difference in RANK expression in the peripheral blood of the JIA patients compared with PBMC from controls.

RANK⁺ synovial cells have the phenotype HLA-DR^{hi}CD86^{hi}CD11c⁺ and express the dendritic cell marker DC-SIGN

RANK protein may be expressed on two major types of myeloid cells: dendritic cells (DC) and osteoclasts. We wished to characterize the RANK⁺ cells in the synovial fluid and especially to determine whether these cells were of the dendritic cell type or osteoclasts. Paired SFMC and PBMC were therefore stained for proteins which are highly expressed on dendritic cells or activated macrophages (CD86, HLA-DR and CD11c), for the recently defined DC-specific adhesion receptor DC-SIGN (CD209), and for CD51, a subunit of the vitronectin receptor, which is expressed on osteoclasts. The RANK⁺ synovial cells expressed high levels of CD86, MHC class II (HLA-DR) and CD11c (Fig. 3A). DC-SIGN protein expression was also demonstrated and the majority of synovial RANK⁺ cells expressed DC-SIGN, although at low levels (Fig. 3B). We did not detect any CD51⁺ cells in the synovial samples analysed (data not shown), suggesting that these cells were unlikely to be osteoclasts. Our data do not, however, preclude the expression of RANK on osteoclasts within the bone and cartilage adjacent to the inflamed synovium. As described previously, DC-SIGN expression on peripheral blood cells was at very low or undetectable levels in both controls and JIA patients [31]. The small numbers of polyarticular JIA patients in this study precluded a comparison of this subgroup with those who presented with oligoarticular JIA.

Culture of synovial dendritic cells leads to loss of RANK protein expression

After culture of SFMC or PBMC cells for 1 week in the presence of GM-CSF and IL-4 [32], both SFMC and PBMC yielded a population of dendritic cells which were CD13⁺CD14⁻ and expressed high levels of DC-SIGN protein (Fig. 4A). The myeloid DC derived from peripheral blood also expressed RANK protein (Fig. 4A). However, in synovial DC-like cells, expression of RANK protein decreased on culture (Fig. 4). This fall in RANK expression was demonstrated after culture of DC from SFMC from a total of seven children with JIA, and the

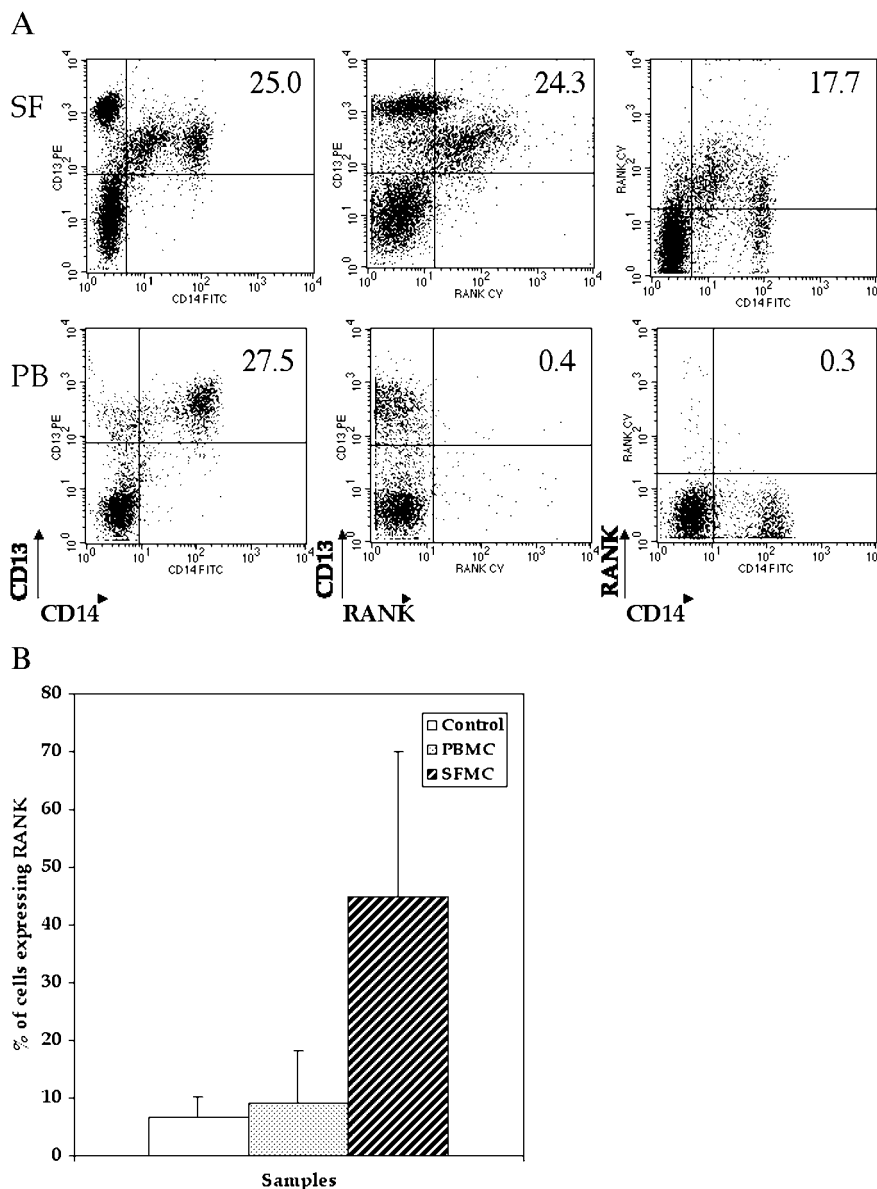


FIG. 2. Flow cytometric analysis of myeloid cells in PBMC and SFMC from patients with JIA ($n=11$). Cells were stained for CD13, CD14 and RANK before analysis on a FACSscan. Plots were generated from data gated by scatter properties to include all myeloid cells. Numbers represent the percentage of events in the top right quadrant of each dot plot. (A) FACS (fluorescence-activated cell sorter) data from a representative JIA patient showing staining of fresh SF (upper panels) and PB (lower panels) myeloid cells for CD13 vs CD14 (left panels), CD13 vs RANK (middle panels) and RANK vs CD14 (right panels). (B) RANK expression as measured by FACS analysis on PBMC from a group of normal healthy children (white bar), and paired samples of PBMC (dotted bar) and SFMC (hatched bar) from JIA patients. Bars represent mean values of RANK⁺ cells within the CD13⁺ population. Error bars represent 1 standard deviation.

decrease was significant ($P=0.04$). On maturation of DC in lipopolysaccharide for 24 h, both conventional DC from peripheral blood and SF DC-like cells showed a fall in the expression of DC SIGN and RANK, as predicted (data not shown) [33].

Discussion

Dendritic cells are the most potent antigen-presenting cells for T cells. Both DC and activated macrophages

may themselves contribute to synovitis by stimulating T cells, but also by producing inflammatory cytokines such as TNF and IL-1. One mechanism which may drive cytokine production by DC is through the binding of RANKL to RANK, a TNF receptor-like surface protein which is expressed on DC. RANK/RANKL interactions may also contribute to increased bone resorption in arthritis. In this study we have shown that RANK and RANKL are overexpressed in mononuclear synovial cells of children with JIA. Periarticular osteopenia has

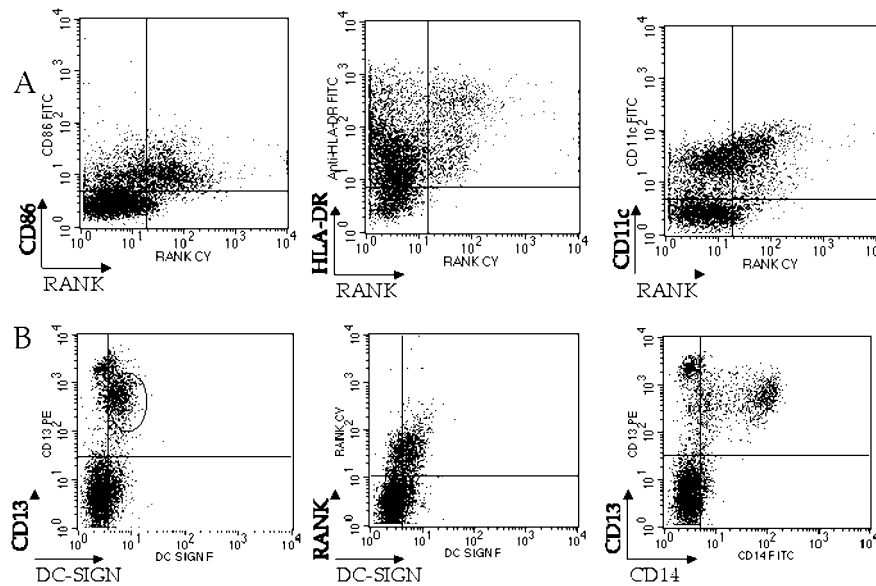


FIG. 3. Phenotyping of the RANK⁺ dendritic-like cells within the SFMC of JIA patients. All plots were generated from data gated by scatter properties to include myeloid cells. (A) Synovial myeloid cells stained for RANK in combination with CD86, HLA-DR or CD11c as shown. (B) Synovial myeloid cells stained for CD13, DC-SIGN, RANK and CD14 as shown.

been demonstrated in children with all subtypes of JIA, and generalized osteoporosis in severe cases is correlated with disease activity, in particular those with systemic-onset JIA [25]. By RT-PCR, we detected RANK mRNA in all but one of the JIA samples, from both blood and SF. However, we also showed significant differences in RANK protein expression between the SF and PB compartments, suggesting that there is a significant degree of post-transcriptional regulation of RANK expression. RT-PCR demonstrated a difference in the level of RANKL in separated cell fractions. Thus, levels of RANKL mRNA expression were higher in JIA patients than controls, and RANKL mRNA was detectable in T cells from both SFMC and PBMC of JIA patients. These data suggest that RANK and RANKL are expressed within the inflamed joint, where they are likely to lead to a local increase in inflammatory and osteoclast activity. The demonstration of RANKL expression in T cells from patients but not controls also suggests that there may be a systemic alteration in RANKL expression, which could contribute to generalized osteoporosis in JIA.

Expression of RANK and RANKL has been demonstrated previously in adult RA patients [17, 19] and some RANK⁺ cells within RA synovium have been shown to express TRAP (tartrate-resistant acid phosphatase) and the calcitonin receptor, indicative of osteoclasts [19, 21]. While our data do not exclude the possibility that the RANK mRNA detected by PCR originates from osteoclasts, we found no expression of the vitronectin receptor (CD51), a marker of osteoclasts.

Another novel finding of this study is the expression of DC-SIGN in SF myeloid cells. Cells expressing RANK protein in SFMC from JIA patients were predominantly of the CD14^{dim} subset. As myeloid dendritic cells mature,

they lose expression of CD14 [34, 35]. We therefore hypothesized that some of the RANK⁺ cells may be immature dendritic cells. Staining for the DC-specific receptor DC-SIGN confirmed that the majority of RANK⁺ cells expressed low levels of DC-SIGN. These RANK⁺ DC-SIGN⁺ cells also expressed high levels of CD86, an important costimulatory molecule, as well as MHC class II (HLA-DR) and the CD11c complement receptor, but low levels of CD83 and CD80. This

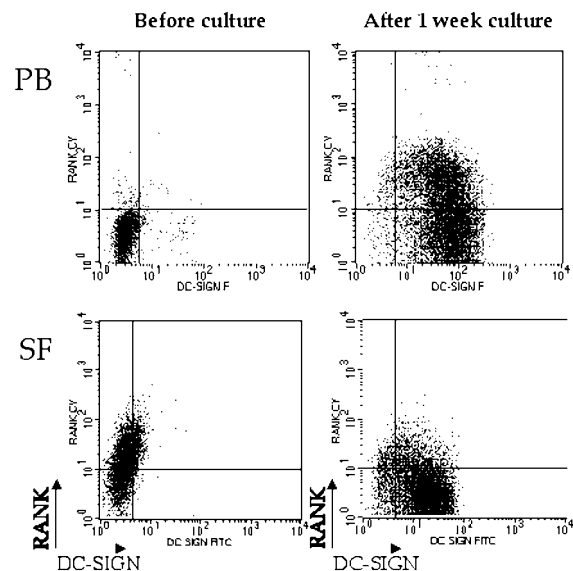


FIG. 4. Flow cytometric analysis of cells from PB or SF before and after 1 week of culture in GM-CSF and IL-4. Cells were stained for RANK and DC-SIGN and analysed by gating on CD13⁺ myeloid cells. Plots represent cells before culture, (left panels) and after 1 week of culture (right panels) as shown.

phenotype is typical of immature myeloid-derived dendritic cells [35–37]. Our data also suggest that a minority of RANK⁺ cells (with a CD14^{hi} phenotype), may be activated macrophages, and that DC-SIGN can also be expressed on such macrophages.

Culture of PBMC and SFMC showed that the DC-like cells from the inflamed joint respond differently to GM-CSF and IL-4 compared with PB myeloid DC. Culture of PBMC yielded a population of CD13⁺CD14⁻ cells, the majority of which expressed high levels of DC-SIGN and RANK protein. In contrast, the DC-SIGN⁺CD13⁺CD14⁻ cells cultured from SFMC expressed low levels of RANK. It is possible that RANK⁺ cells within the SF are dependent upon the presence of soluble RANKL, and that when cultured in the absence of added RANKL, these cells undergo apoptosis *in vitro*.

The demonstration that RANK and RANKL are overexpressed in juvenile arthritis has implications for our understanding of this group of debilitating diseases. The osteoporosis of patients with JIA can lead to low peak bone mass, with consequences in later life. RANK and RANKL, in particular circulating soluble RANKL protein, may contribute to this process. Several inflammatory cytokines which contribute to bone loss, such as TNF, IL-1, IL-6 and IL-17, have an effect via the RANK/RANKL pathway by up-regulating RANK expression on osteoclasts or RANKL production by T cells [38, 39]. In this context, it would be of particular interest to study RANK and RANKL expression in children with systemic-onset JIA (SOJIA), as these patients often develop severe osteoporosis. These were not included in the present study because our management of SOJIA only rarely includes joint aspiration.

Cells with features typical of dendritic cells have been demonstrated within both the SF and synovial tissue of patients with JIA and RA [40–42]. Mature DC may play a central role in the presentation of antigens to T cells and the production of inflammatory cytokines. The demonstration that DC that have been genetically modified to express the anti-inflammatory cytokines can have an effect on distant sites of arthritis when injected into one joint suggests that DC may be a valuable tool for delivery of new therapies in the form of gene therapy [43, 44].

However, taken together, our data may suggest an alternative possibility for the role of the DC which we have identified in JIA synovial samples. These DC have an immature phenotype and express RANK: immature DC have been shown to be able to induce regulatory T cells (T_{reg}), which may suppress an immune response, or even to induce tolerance [45, 46]. In addition, recent data suggest that RANK/RANKL interactions may be critically involved in the generation of such T_{reg} [46]. Thus it is possible that the immature RANK⁺ DC we have identified may play an immunoregulatory role in JIA. This is of particular interest, given the mounting evidence for immunoregulation in oligoarticular JIA, in which approximately 60% of children go into spontaneous prolonged remission, and in whom 'regulatory'

cytokines [5, 47] and cells suggestive of T_{reg} have been identified from the joint [48]. From this hypothesis, we predict that synovial DC obtained from mild, remitting oligoarticular JIA should be able to support the generation of regulatory T cells, while those from severe polyarticular cases of JIA should be more proinflammatory. A comparison of these two groups was not possible from our study because of small numbers, but such a comparison is now planned.

In conclusion, we have extended the available data by demonstrating a population of DC-SIGN⁺ DC-like cells, which express RANK, within the joints of children with JIA. The mechanisms which favour survival of DC within the inflamed joint are multifactorial. Cells which express DC-SIGN protein may be able to enter the inflamed site selectively, through increased binding to ICAM-2 under flow conditions [31]. In addition, the interaction between RANK and RANKL can deliver activating and survival signals to DC [14]. Our study provides the first evidence that the RANK/RANKL pathway is altered as part of the inflammatory process which leads to destructive synovitis in children, and a suggestion that cells of a dendritic cell type may be involved in this process, in part through their expression of the RANK protein. An understanding of the regulation of RANK and RANKL expression may lead to the design of novel adjunct therapies to block inflammation and bone damage in juvenile arthritis.

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