Low density of CD1+ cells in the synovial tissue of patients with rheumatoid arthritis

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Abstract

Objective

CD1 molecules present microbial and self glycolipid antigens to a defined T cell subset with features of natural killer cells. CD1 molecules are up-regulated by inflammatory stimuli such as GM-CSF, and we would expect to find increased CD1 expression in the synovium of patients with rheumatoid arthritis (RA) as compared to osteoarthritis (OA). This study was initiated to compare the density of CD1a+, CD1b+, and CD1c+ synovial cells in RA and OA patients.

Methods

Expression of CD1a+, CD1b+, and CD1c+ molecules in synovial tissue was assessed by semiquantitative immunohistochemistry. For comparison, serological, functional, and typical immunohistochemical markers of inflammation were detected.

Results

Although patients with RA as compared to OAhad highly significantly increased signs of inflammation, the density of CD1a+, CD1b+, and CD1c+ synovial cells was similar. This was also true for the density of CD1+ cells in relation to that of activated CD163+ macrophages. There was a high correlation between the densities of CD1a,b,c positive cells, which suggests the existence of similar regulatory pathways. In a combined analysis of RA and OA patients, there existed a negative association between prior NSAID therapy and the density of CD1a+, CD1b+, and CD1c+ synoviocytes in relation to CD163+ macrophages. This is interesting because a similar immunosuppressive aspect of NSAID has never been shown before and this might represent a hitherto unrecognized immunosuppressive aspect of NSAID.

Conclusion

Considering the high synovial inflammation in patients with RA, the densities of CD1a+, CD1b+, and CD1c+ synovial cells were low compared to patients with OA. Further studies in RA patients are needed to clarify whether a defect in CD1 regulation may exist. Such a defect may lead to an insufficient immune response against microbial glycolipids, which would support smoldering or repeated inadequately responded infection.

Key words

Rheumatoid arthritis, osteoarthritis, CD1a, CD1b, CD1c.

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Introduction

The role of microbial antigens in the pathogenesis of rheumatoid arthritis (RA) is still a matter of debate (1-5). *Mycoplasma*, *Proteus*, *Chlamydia*, *Escherichia* and, particularly, atypical mycobacteria may play a role: e.g., responsiveness of synovial fluid lymphocytes to mycobacterial antigens such as the 65 kD heat-shock protein was found early in patients with RA as well as in patients with other forms of chronic inflammatory arthritis (6). In a patient with RA, γ/δ T cell clones were isolated from the synovial fluid which responded specifically to mycobacterial antigens without MHC restriction (7). The limited receptor repertoire of this subset of T cells and activation by mycobacterial antigens resistant to proteases suggest that they may recognize non-peptide antigens (8). Presentation of non-peptide microbial antigens may be an important factor in the activation of the immune system, particularly restricted T cell subsets. The question remains how non-peptide antigens may be presented to Tcells.

Until recently, it was believed that T cells recognize exclusively protein-derived antigens by the MHC class I and II antigen-presenting molecules. This system was thought to be sufficient for effective T cell responses against extra- and intracellular pathogens. This paradigm has now changed with the realization that the immune system is able to present lipid antigens to T cells via CD1 molecules (9). There are two groups of human CD1 molecules: CD-1a, CD1b, and CD1c form group I, and CD1d forms group II (10). CD1 molecules are β2-microglobulin-associated proteins that are structurally related to class I MHC molecules, but have a hydrophobic antigen-binding pocket that appears to be specialized for binding the hydrocarbon chains of lipids (11). Antigenic lipids are derived from extracellular sites and are processed to be presented on the cell surface (12,13). In particular, mycobacterial but also self glycolipids are presented by CD1 molecules (12,14,15).

It was the aim of this study to investigate the tissue density of group I CD1+ synoviocytes in patients with RA and osteoarthritis (OA). Typical functional and histological inflammatory markers were evaluated to investigate their relationship with the density of CD1+ cells. Furthermore, the influence of prior medication on the density of CD1+ cells was also investigated.

Patients and methods

Patients and controls

From a total group of 79 patients with OA and 95 patients with RA who had undergone knee joint replacement surgery, we randomly included 9 RAand 8 OA Caucasian patients in a first study (Table I). These patients markedly differed in their serological and histological signs of inflammation (Tables I and II, for techniques see below). In a second confirmatory study, we randomly investigated 23 RA and 28 OA Caucasian patients drawn from the same total group (excluding patients from the first study), in order to study the density of CD1c+ cells (for technique, see below) (Table I). Similarly, the serological and histological signs of inflammation in the OA and RA patients from the second study were very different (Table II, techniques see below). NSAID were administered for a minimum of 4 weeks before surgery. In part, these patients were also used in another study (16). RApatients fulfilled the American College of Rheumatology criteria for RA (17). RA and OA patients were matched for age and gender. The patients were informed about the purpose of the study and gave their written consent. The study was approved by the Ethical Committee of the University of Regensburg. Serological parameters of inflammation such as the erythrocyte sedimentation rate were measured by standard techniques.

Synovial tissue preparation

Synovial tissue samples were obtained immediately after opening the knee joint capsule. The preparation of the tissue for histology has been previously described (18,19). Briefly, one piece (≈ 9 cm^2) of synovial tissue was dissected. Fat tissue and tissue with a large number of vessels were removed. Eight pieces (≈16 mm^2) were loaded into 8 superfusion chambers (see below) and

Table I. Basic characteristics of patients with osteoarthritis and rheumatoid arthritis in the first and second study. Data of patients in the second confirmatory study are given in between {}. Data are expressed as means ± SEM and ranges are shown in brackets.

	Osteoarthritis	Rheumatoid arthritis
Number	8 {28}	9 {23}
Age (yrs.)	61.6 ± 4.6 [41 - 75] {62.3 ± 1.6 [52 - 68]}	69.9 ± 1.6 [49-88] {65.4 ± 2.4 [39-84]}
Duration of disease (yrs.)	8.2 ± 0.8 [5 - 12] {7.6 ± 0.6 [5 - 11]}	4.5 ± 1.0 [1 - 8] {13.7 ± 1.6 [3 - 27]*}
Gender (f/m)	6 / 2 {23 / 5}	7 / 2 {20 / 3}
ESR (mm/1st hr)	11.0 ± 2.6 {16.8 ± 2.3}	34.6 ± 9.5[a] {31.5 ± 5.6*}
Medication[c]		
Prednisolone[b]	0 {1}	5 {15}
Methotrexate	0 {0}	2 {3}
Sulfasalazine	0 {0}	1 {2}
Azathioprine	0 {0}	1 {0}
Gold compounds	0 {0}	1 {0}
Leflunomide	0 {0}	0 {4}
NSAID	4 {12}	7 {13}

[a]p = 0.054, *p < 0.05 for the comparison of the two groups.
[b]The prednisolone dose in RApatients ranged between 2.5 - 15.0 mg/day in both studies.
[c]The duration of DMARD therapy is not exactly known.
ESR: erythrocyte sedimentation rate

8 pieces (≈ 1 cm^2) pieces of the same synovial area were used for histology. The samples intended for the hematoxylin-eosin (HE) and alkaline phosphatase anti-alkaline phosphatase (APAAP) staining were immediately placed in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and then quick-frozen by floating on liquid nitrogen. The tissue samples used for the detection of sympathetic nerve fibers were fixed for 12 to 24 hours in phosphate buffered saline (PBS) containing 3.7% formaldehyde and then incubated in PBS with 20% sucrose for 12 to 24 hours. The tissue was then bedded in Tissue-Tek and quick-frozen. Each patient's samples of synovial tissue were stored at -80°C.

Table II. Markers of inflammation in patients with osteoarthritis and rheumatoid arthritis in the first and second study. Data of patients in the second confirmatory study are given in braces. Data are given as means ± SEM.

	Osteoarthritis	Rheumatoid arthritis	p-value
Superfusion			
Superfusate IL-6_{2hr} (pg/ml)	92.4 ± 15.5 {168.8 ± 18.7}	105.9 ± 8.9 {281.4 ± 51.0}	n.s. {n.s.}
Superfusate IL-8_{2hr} (pg/ml)	40.5 ± 7.8 {87.3 ± 8.7}	86.3 ± 16.2 {252.8 ± 45.9}	0.036 {0.001}
Histology			
Lining layer thickness (cells)	2.7 ± 0.3 {1.9 ± 0.1}	7.0 ± 0.3 {4.6 ± 0.5}	0.040 {< 0.001}
CD3+ Tlymphocytes/mm^2	7.8 ± 1.1 {25.3 ± 4.4}	42.4 ± 10.8 {61.7 ± 16.6}	0.005 {0.038}
CD163+ macrophages/mm^2	52.8 ± 8.2 {56.8 ± 7.7}	115.2 ± 26.1 {100.9 ± 17.8}	0.024 {0.047}
TH+ sympathetic nerve fiber/mm^2	2.3 ± 0.3 {2.0 ± 0.3}	0.5 ± 0.1 {0.6 ± 0.1}	0.001 {< 0.001}
Overall cellularity (cells/mm^2)	791 ± 45 {1289 ± 88}	1295 ± 115 {2064 ± 164}	0.001 {< 0.001}
Overall vascularity (vessels/mm^2)	17.7 ± 1.6 {28.2 ± 3.0}	24.2 ± 2.2 {31.9 ± 3.6}	0.046 {n.s.}

IL: interleukin; TH+: tyrosine hydroxylase positive (key enzyme in norepinephrine production and a marker of sympathetic nerve fibers).

Histological evaluation of inflammation and determination of synovial innervation

The histological evaluation procedure has been described in an earlier study (18). Briefly, the frozen tissue samples were cut into 6-8 µm thick sections and cell density and lining layer thickness were evaluated using a standard HE staining of approximately 45 sections. At a magnification of 400x, the extent of the lining layer thickness was determined by averaging the number of cells in a lining layer cross section at 9 different locations. The cell density in the synovial tissue was determined by counting all stained cells in 17 randomly selected high power fields (400x) and expressed per mm^2. In order to determine the number of T-cells (CD3, clone UCHT 1, Dako, Hamburg, Germany), macrophages (CD163, clone BerMAC, Dako, Hamburg, Germany), capillary vessels (collagen IV, clone CIV 22, Dako, Hamburg, Germany), CD1a+ cells (clone NA1/34, Serotec, Kidlington, U.K.), CD1b+ cells (clone 4.A7.6, DPC Biermann, Bad Nauheim, Germany), and CD1c+ cells (clone L161, Serotec, Kidlington, U.K.) in the synovial tissue of each patient, eight cryosections were investigated using APAAP staining and the number of identified structures was averaged from 17 randomly selected high power fields (400x) and expressed per mm^2.

Since the presence of tyrosine hydroxylase-positive nerve fibers (TH, the key enzyme for NE production in sympathetic nerve endings) is an excellent marker of synovial inflammation, the density of the synovial sympathetic nerve fibers was determined as described earlier (18,19). Briefly, six to

eight 7-9 µm thick cryosections were used for immunohistochemistry with primary antibodies against TH (polyclonal, Chemicon, Temecula, USA). A Cy3-labeled secondary antibody (Dianova, Hamburg, Germany) was used to obtain immunofluorescent staining. The numbers of stained nerve fibers per mm^2 were determined by averaging the number of stained nerve fibers with a minimum length of 50 µm (indicated by a small micrometer eyepiece) in 17 randomly selected high power fields (400x), respectively.

Superfusion technique of synovial tissue and cytokine analysis in the superfusate

As described in detail for spleen slices (20,21), we used a microsuperfusion chamber apparatus to superfuse pieces of synovial tissue with culture medium (RPMI 1640, 25 mM HEPES, 5% FCS, 1% Pen/Strep, 30 µM mercaptoethanol, 0.57 mM ascorbic acid, 1.3 mM calcium, all additions from Sigma, Deisenhofen, Germany). These superfusion chambers had a volume of 80 µl. Superfusion was performed for 2 hours at a temperature of 37°C and a flow rate of 66 µl/min (one piece per chamber, 24 chambers in parallel). At 120 min the superfusate was collected in order to measure the cytokine concentrations in a fraction of approximately 1 ml (collected over 15 min). Human IL-6 and IL-8 (detection limit in the two assays: <2 pg/ml, Endogen, Boston) were determined by enzyme immunometric assay. Since in the superfusion experiments TNF was not measurable in about 50% of the patients, we did not include TNF as a read-out parameter.

Presentation of the data and statistical analysis

All data are given as the mean ± SEM. Correlations were calculated by Spearman Rank Correlation analysis (SPSS/PC, Advanced Statistics, V10.0.1, SPSS Inc., Chicago). Group medians were compared by the non-parametric Mann-Whitney test (SPSS). $p < 0.05$ was the significance level.

Results

Histological inflammation in patients with RA and OA

As expected, cytokine superfusate concentrations of IL-8 (not significant for IL-6), and lining layer thickness, CD3+ T cell density, CD163+ macrophage density, and overall cellularity were significantly higher in patients with RA as compared to OA (Table II). In contrast, density of TH+ sympathetic nerve fibers were significantly lower in RA as compared to OA (Table II). Apart from erythrocyte sedimentation rate (Table I), these functional and histological parameters indicate that inflammation was markedly higher in RA as compared to OA patients (Table II). This is true for patients in the first and second confirmatory study.

CD1+ cells in patients with OAand RA

Figure 1 demonstrates representative examples of CD1 staining using the APAAPmethod. It is demonstrated that the antibodies used show a cell specific

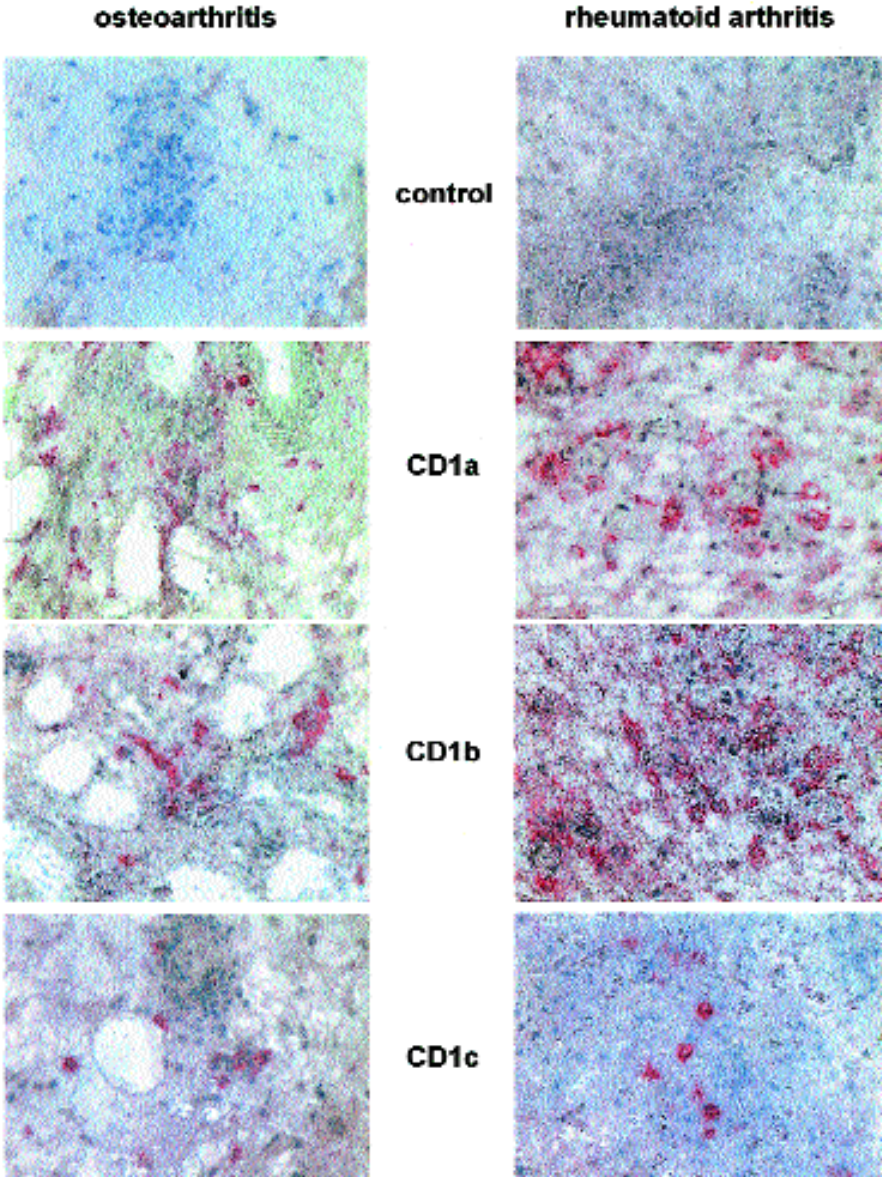

Fig. 1. Immunohistochemistry for CD1a, CD1b, and CD1c in synovial tissue of patients with osteoarthritis (**left**) and rheumatoid arthritis (**right**). Antibody directed against CD1a,b,c were used together with Mayer's hematoxylin for counterstaining. The panels demonstrate representative high power fields, x 400. In the analyses, the number of identified positive cells was averaged from 17 randomly selected high power fields (400x) and expressed per mm^2.

staining which was not observable when using IgG1 or IgG2 primary control antibodies (Fig.1). Although the markers of inflammation were much higher in the RA as compared to the OA patients, density of CD1a+, CD1b+, and CD1c+ cells demonstrated no significant difference between OA and RA patients in either study (Table III). This was surprising since the density of macrophages, which normally express CD1 molecules, was significantly increased in the RAas compared to the OApatients (Table II).

In order to directly demonstrate the density of CD1+ cells in relation to the density of CD163+ macrophages, the ratio of these two parameters for every patient was calculated (100 x density of CD1a,b,c+ cells divided by the density of macrophages, unitless ratio). As shown in Figure 2A, in the first study this particular ratio for CD1a tended to be lower in RA as compared to OA, and the ratio for CD1c was significantly lower in RAthan in OA. If we calculate the density of CD1a,b,c cells in relation to overall cellularity, no such difference existed (data not shown). In the second confirmatory study with a larger number of patients, RA and OA patients did not significantly differ in the density of CD1c in relation to the density of CD163+ macrophages (Fig. 2B).

In order to further investigate the correlation of the density of cells expressing group I CD1 molecules, we used Spearman rank correlation (Table IV). This analysis revealed highly significant correlations between the density of CD1a,b,c positive cells in all patients and in patients with RA and OA. With respect to the combination CD1a+ vs. CD1c+ and CD1b+ vs. CD1c+, this did not reach the significance level in OA due to the small number of patients (Table IV). This analysis may indicate that upregulation of a certain CD1 molecule is often accompanied by the upregulation of other CD1 molecules from the same group, which suggests similar regulatory pathways.

CD1+ cells in patients with OA and RA in relation to prior medication

Since there was no detectable difference in the density of CD1a, b, c positive cells between OA and RA (Table III, Fig. 2), we constructed a new total group of 17 (first study) and 51 patients (second study) and compared those with versus those without prior NSAID medication. Interestingly, patients with prior NSAIDs demonstrated a decreas-

Table III. Density of CD1 positive cells in synovial tissue of patients with osteoarthritis and rheumatoid arthritis in the first and second study. Data of patients in the second confirmatory study are given between {} (only CD1c investigated). Data are given as means ± SEM.

	Osteoarthritis	Rheumatoid arthritis
CD1a+ cell density (cells/mm^2)	3.0 ± 1.9 [0.0 - 15.9]	5.7 ± 3.8 [0.0 - 32.6]
CD1b+ cell density (cells /mm^2)	0.6 ± 0.3 [0.0 - 2.1]	1.3 ± 0.8 [0.0 - 6.0]
CD1c+ cell density (cells /mm^2)	1.9 ± 0.8 [0.0 - 7.0] {1.4 ± 0.2 [0.0 - 4.3]}	0.9 ± 0.3 [0.0 - 2.7] {1.9 ± 0.5 [0.0 - 10.5]}

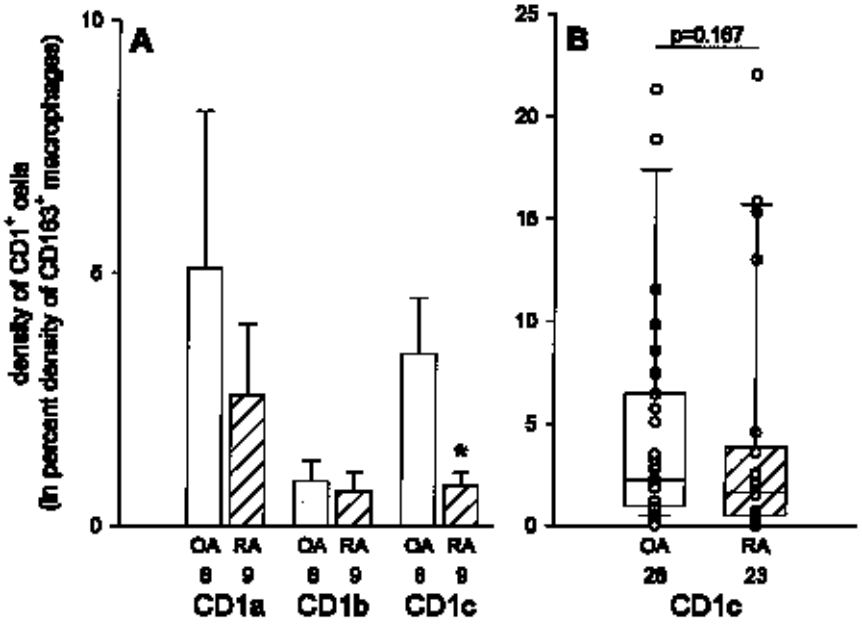

Fig. 2. Density of CD1+ cells in relation to CD163+ macrophages. (**A**) The panel gives the results for CD1a, CD1b, and CD1c in the first study. Data are expressed as means ± SEM. *p < 0.05 vs. osteoarthritis. (**B**) CD1c data are given for the second confirmatory study. Box plots represent the 10th, 25th, 50th (median), 75th, and 90th percentile(**A**. Furthermore, the open circular symbols (❍) give the results for individual patients. For both panels, the number of patients with osteoarthritis (white bars) and rheumatoid arthritis (hatched bars) are given below the x-axes.

Table IV. Correlation of the density of CD1 positive cells in all patients and in patients with osteoarthritis (OA) and rheumatoid arthritis (RA) in the first study. No such correlation analysis was carried out in the second confirmatory study due to missing CD1a and CD1b determinations. Rank correlation coefficients and respective p-values are given for all patients and for the patients in the two disease subgroups.

	CD1a+ cell density	CD1b+ cell density
CD1b+ cell density	all: R_{Rank} = 0.872, p < 0.001 RA: R_{Rank} = 0.869, p = 0.002 OA: R_{Rank} = 0.868, p = 0.005	—
CD1c+ cell density	all: R_{Rank} = 0.612, p = 0.009 RA: R_{Rank} = 0.714, p = 0.031 OA: R_{Rank} = 0.440, n.s.	all: R_{Rank} = 0.635, p = 0.006 RA: R_{Rank} = 0.755, p = 0.019 OA: RR_{ank} = 0.517, n.s.

ed density of CD1a+ and CD1b+ cells (Fig.3A). When the density of CD1-a,b,c cells was expressed in relation to the density of CD163+ macrophages, patients with prior NSAIDs demonstrated a decreased density of CD1a+, CD1b+, and CD1c+ cells (Fig. 3B). In the first study no patient had a specific cyclooxygenase (COX) inhibitor, whereas in the confirmatory study 4 patients were treated with rofecoxib. Patients with versus those without rofecoxib did not differ in the density of CD1c+ cells nor in the density of CD1c+ cells in relation to CD163+ macrophages (data not shown). Furthermore, patients with unspecific COX inhibitors versus patients with rofecoxib did not differ in the density of CD1c+ cells nor in the density of CD1c+ cells in relation to CD-163+ macrophages (data not shown). In the first study and in the confirmatory study, no other differences were found in the comparisons of patients with/ without other typical DMARDs (methotrexate, sulfasalazine, azathioprine, and gold compounds). In the confirmatory study, patients without versus those with prior leflunomide treatment tended to present an increased density of CD1c+ cells (1.7 ± 0.3 versus 0.8 ± 0.5 CD1c+ cells/mm^2, $p = 0.065$; 5.6 ± 1.4 vs. 1.2 ± 0.6 CD1c+ cells in % of macrophages, $p = 0.065$).

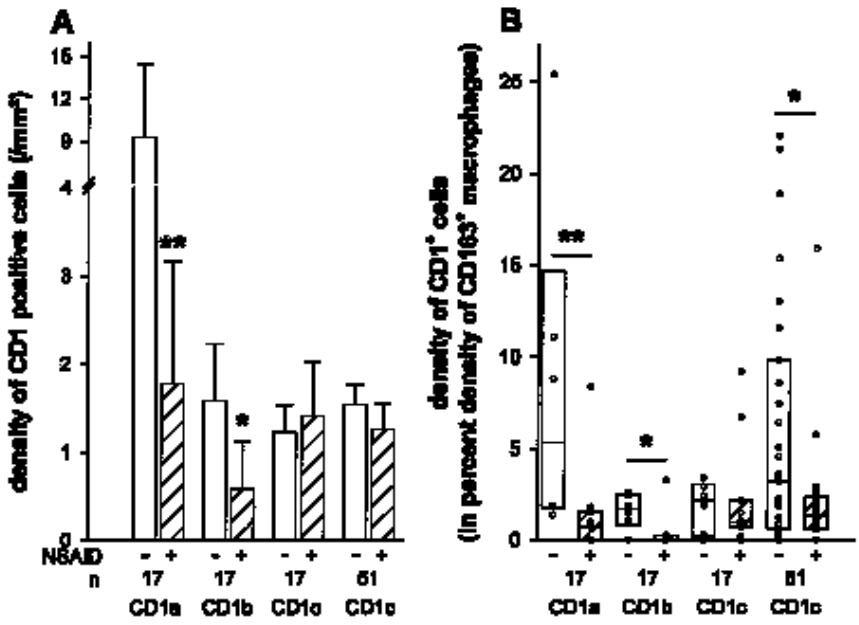

Fig. 3. Density of CD1+ cells in relation to prior therapy with NSAIDs (+) or without NSAID (–). (**A**) The panel gives the results for the density of CD1a+, CD1b+, and CD1c+ cells from both studies (n = 17 refers to the first study and n = 51 to the confirmatory study). Data are expressed as means ± SEM. (**B**) The panel shows the density of CD1a,b,c+ cells in relation to the density of CD163+ macrophages in form of box plots (unit = % of CD163+ macrophages). In these plots, the 25th, 50th (= median), and 75th percentiles are given. Furthermore, the open circular symbols (❍) show the results for individual patients. **p < 0.01, *p < 0.05 for the comparison of patients with NSAIDs versus those without NSAIDs. For both panels, the numbers of all investigated patients are given below the x-axes.

Discussion

This study demonstrated that patients with RAin comparison to patients with OAhad very similar densities of CD1+ cells, although inflammation was much more marked in the RA patients. Generally, CD1 expression was rarely found in the synovial tissue of patients with OA and RA. A density of 0.6 to 5.7 CD1+ cells per mm^2 is low in relation to 52.8 to 115.2 activated macrophages per mm^2. The relatively low number of CD1+ cells corroborates two recent studies in RA patients (22, 23), although these two studies did not quantify the density of CD1+ cells in a large number of high power fields, did not compare synovial density in RA patients with OA patients, and did not compare the inflamed synovial area in RA with the less inflamed synovium in OA. Cauli *et al*. compared the density of CD1+ cells in RAsynovium with the epidermis and dermis in psoriasis patients (22). This interesting investigation revealed significantly increased numbers of CD1a+, CD1b+ and CD-1c+ cells in the inflamed skin as compared to inflamed synovium. The authors concluded that different subsets of antigen-presenting cells are involved in the pathogenesis of dermatitis and synovitis. Under additional consideration of less inflamed OA tissue, we support the finding of low numbers of CD1+ cells in inflamed RA synovium. The question arises as to why in RA patients the density of group I CD1+ cells is surprisingly low in the presence of activated macrophages and other cells.

Since the *in vitro* stimulation of monocytes/macrophages leads to the upregulation of different group I CD1 molecules (10,12,24), we would expect an increased density of cells bearing group I CD1 molecules in inflamed tissue. For example, GM-CSF is a stimulator of CD1 expression (10) and the same cytokine was found to be significantly up-regulated in RA synovium (25, 26). At the moment, the reason for the low numbers of CD1-expressing cells in RA patients is completely unclear. One may speculate that there is a defect in CD1 upregulation in RA patients which remains to be elucidated. Such a defect would lead to an insufficient immune response against microbial glycolipids, thus supporting a smoldering or repeated inadequately responded infection.

Another explanation for the low numbers of CD1 expressing cells in RA patients could lie in the fact that antigenic glycolipids do not represent a pathogenetic element in this disease. However, several reports have mentioned that glycolipids may play a role in at least a subset of patients with RA because peripheral cells can be better activated by glycolipids than in healthy controls (27, 28). Furthermore, independent reports in RA patients demonstrated elevated antibody titers against different glycolipids (29-31). Glycolipids may function as autoantigens, a phenomenon which has not been con-

vincingly demonstrated in RA. Nevertheless, one would expect increased numbers of CD1+ cells in inflamed RA synovial tissue. This may be another indication that group I CD1 expression is low in these patients. Interestingly, in our study there existed a negative association between prior NSAID therapy and the expression of CD1a, CD1b, and CD1c. Thus, prior NSAID therapy may also play a role in the low numbers of group I CD1+ cells in the synovium. Furthermore, in the confirmatory study there was no difference in the density of CD1c+ cells in patients using unspecific COX versus specific COX-2 inhibitors. We may summarize that COX inhibitors – whether specific or unspecific – seem to downregulate group I CD1 molecules. This is interesting because a similar immunosuppressive aspect of COX inhibitors has never been shown. In contrast, others have demonstrated in T cells that unspecific or specific COX inhibitors are not immunosuppressive (32). Indeed, this might be a hitherto unrecognized immunosuppressive aspect of NSAIDs. In our study, such a negative association was not observed for DMARDs (particularly not for methotrexate). Other studies in various disease entities have demonstrated a decrease in the density of CD1 positive cells after azathioprine treatment (33,34) and an increase after FK-506 treatment (35). Since only one patient in our study was treated with azathioprine and none with FK506, we are unable to confirm this interesting association.

In conclusion, this study confirms the low expression of group I CD1 molecules in the inflamed synovial tissue of patients with RA. Furthermore, it is suggested that there is no substantial difference in the density of group I CD1+ cells in OA as compared to RA. At present the low expression of group I CD1 molecules cannot be sufficiently explained and thus deserves further intensive investigation.

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