Osteoarthritic articular chondrocytes stimulate autologous T cell responses *in vitro*

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Abstract

Objective

To clarify the presence of specific T cell immune response to autologous chondrocytes in patients with osteoarthritis (OA).

Methods

Peripheral blood mononuclear cells obtained from OA or post-traumatic patients were co-cultured with irradiated autologous chondrocytes, and their proliferative response was assessed using ^{3}H-thymidine incorporation. Expression of HLA-class II molecules was also assessed on chondrocytes by immunohistochemistry or flow cytometry.

Results

T cell responses to autologous chondrocytes in OA yielded a significantly greater mean stimulation index (6.35 ± 1.63) compared to controls (1.21 ± 0.09, $p < 0.01$). This response was partially blocked by antibodies against HLA class I, class II, CD4 or CD8. Increased expression of HLA-DP, –DQ, and –DR was observed.

Conclusion

This study showed the autologous T cell-stimulating property of OA chondrocytes in vitro. *The elucidation of the autoimmune responses may contribute to the understanding of immune-mediated mechanisms in OA.*

Key words

Osteoarthritis, chondrocytes, T cells, autoreactivity.

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Introduction

Osteoarthritis (OA) is a chronic disease in which multiple risk factors such as aging, genetic factors, joint injury or skeletal deformity contribute to the pathogenesis (1). Histological examination of OA cartilage revealed surface fibrillation and loss of the extracellular matrix, including proteoglycans (2). Even though synovitis occurs in the OA joints, it is milder than that in rheumatoid arthritis (RA) (2). Furthermore, systemic inflammatory signs are apparently absent in patients with OA. Thereby, OAhas been considered to be a non-inflammatory disease. However, recent studies have documented that cellular components of the OA synovium did include activated T cells, B cells and monocytes/macrophages, which were thought to be immunocompetent (3, 4). In addition, we previously reported that T cells infiltrating the OA synovial tissue included clonally expanded populations, suggesting the presence of an antigen-driven T cell response (5). These data may indicate autoreactive T cells in OA.

Regarding immunogenic antigens in OA, various proteins derived from cartilage or chondrocytes, e.g. collagens and proteoglycans, are recognized as being a target of specific immune responses in OA patients (6-8). More recently, we found that cartilage intermediate layer protein (CILP), a protein with increased expression in older people or in patients with OA, is also a target autoantigen in a subset of patients with OA as well as RA (9). Moreover, some of these cartilage-related antigens elicit arthropathy in animal models (9-12). Most of these proteins are reported to be secreted from articular chondrocytes, and therefore it is suggested that a specific T cell immune response to the chondrocyte-related antigens may occur in the vicinity of the cartilage in the OAjoint.

T cells recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules on antigen presenting cells (APC) by means of the T cell receptor (TCR) specific for their antigens. In addition to the signals transmitted through the TCR-antigen-MHC complex, co-stimulatory signals are required for full activation of T cells, which are delivered through surface molecules such as CD80 (B7) or CD86 (B7-2) on APC (13,14). In this regard, a significant proportion of chondrocytes from both RA and OA patients has been reported to express MHC class II molecules, especially after stimulation with IFNγ (15). In addition, it has been suggested that normal articular chondrocytes are able to process and present antigens to T cells (16-18). However, the significance of such "antigen presenting" capacity in OAchondrocytes is still unclear.

In this present study we investigated the T cell immune response against autologous articular chondrocytes.

Materials and methods

Patients and specimens

Thirteen patients with OA (age 53-80 years; mean 69.7, M/F = 1/12) were enrolled in this study on T cell responses to chondrocytes. Normal controls consisted of 7 patients with traumatic hip fracture (age 59-90 years, mean 73.1, M/F = 1/6) with no history of joint injury or rheumatic diseases. Articular cartilage obtained from traumatic patients showed no abnormal findings macroscopically, microscopically or radiologically, and thus were classified as "normal". Articular cartilage tissue was obtained at the time of joint surgery, and heparinized peripheral blood was collected at the same time. For immunocytochemistry, a different panel of patients made up of 5 with OA (age 58-75; mean, 67.0), 5 with RA (age 55-79; mean, 66.4) and 5 with traumatic fractures (age 55-88; mean, 74.6) was studied. The diagnoses of OAand RAwere made according to the published criteria (19, 20). The OA patients were treated by the temporary use of non-steroidal anti-inflammatory drugs, and all of them were scored as having grade IV OA by the Kellgren & Lawrence classification (19). The RA patients were administered at least one of the following disease-modifying antirheumatic drugs: gold, bucillamine, salazosulfapyridine and/or D-penicillamine, and all of them had stage IV RA according to Steinblocker's classification (21). The samples were ob-

tained with informed consent, and the study protocol was approved by the ethical committee of St. Marianna University School of Medicine.

Chondrocyte culture

Articular cartilage specimens were obtained from knee joints of the OA patients undergoing total joint replacements and from hip joints of fracture patients undergoing prosthetic surgery. Each cartilage sample was carefully freed of synovial and subchondral tissue by cutting into sections, and was then minced. The extracellular matrix was digested for 15 hours at 37ºC with 1 mg/ml type I collagenase (SIGMA, St. Louis, USA) in DMEM/FBS medium (GIBCO BRL, New York, USA). . The resulting cell suspensions were filtered through a nylon sieve with a pore size of 75 μm, washed twice in phosphate buffered saline, and further expanded in the conditioned medium (DMEM supplemented with 2.05 mM L-Glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin).

Separation of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by the standard density gradient centrifugation method using Ficoll-Paque Research Grade (Amersham Pharmacia Biotech AB, Uppsala, Sweden). T lymphocytes were isolated from the PBMC by a T-cell enrichment column kit (R&D, Minneapolis, USA).

Autologous lymphocyte response to chondrocytes

The proliferative response of PBMC and T cells to chondrocytes was assayed according to a previously described method with some modification (22, 23). Briefly, chondrocytes from the primary cultures were seeded at 5 x 10^4 cells/well in 0.1 ml of RPMI/10%FBS into type I collagen-coated 96-well microplate wells (Becton Dickinson, NJ, USA). After 24 hours, the chondrocytes were irradiated at 4000 rad by a roentgenoradiator (Hitachi Medical Co., Yokohama, Japan) (24). After washing, autologous PBMC or T cells (1 x 10^6 cells/well in 0.1 mL in RPMI/FBS) were added to the chondrocytes. The mixed cultures were incubated for 4 days at 37ºC in a humidified atmosphere of 95% air and 5% CO_2, since in preliminary experiments we observed a significant difference between the results from OA and normal chondrocytes at this time point (data not shown).

After the culture period, one microcurie of ^{3}H-labeled thymidine (NEN Life Science Products, Boston, USA) was added to each well during the final 18 hours of incubation. Cells were then harvested semi-automatically by a Harvester 9600 (TOMTEC, Connecticut, USA), and the incorporation of ^{3}H-labeled thymidine was assayed using a beta scintillation counter, MicroBeta TriLux (WALLAC, Turku, Finland). For each sample, the counts per minute (cpm) values obtained from cultures of PBMC or T cells alone were used as background. The stimulation index (SI) was calculated by dividing the cpm from co-cultures by the cpm of the background values. For the blocking experiments, monoclonal antibodies were added to the coculture simultaneously. The antibodies used were anti-CD4, anti-CD8, anti-HLA class I, anti-HLA class II-DP, -DQ, -DR, and anti-mouse IgG antibosies antibodies (Table I).

Immunocytochemistry

Chondrocytes were seeded onto 8-well chamber slides (Nalge Nunc International Corp., Illinois, USA) at a concentration of 2x 10^4 per well for 72 hours, and then immunolocalization was performed using immunostaining kits [DAKO Catalyzed Signal Amplification System (DAKO, Carpinteria, USA)] according to the following protocol. Chondrocytes on the slides were fixed using 4% formaldehyde in PBS at room temperature for 30 min. After fixation, the cells were pre-incubated with a blocking solution, BlockAce™ (Dainippon Pharmaceutical Company; Osaka, Japan) at room temperature for 1 hour, and then incubated at room temperature for 1 hour with one of the monoclonal antibodies either against HLA-DP, -DQ, -DR, or with an isotype control. The incubated cells were washed twice with PBS containing 0.1% Tween 20 (PBST), and then incubated with a biotinylated rabbit anti-mouse IgG (KPL, Maryland, USA). The chondrocytes were counterstained with Methyl Green (DAKO) and were visualized using diaminobenzidine. Then percentages of positive cells for each antibody were calculated in each group.

Statistical analysis

Comparisons between the groups were performed the Mann-Whitney U test.

Results

T cells respond to autologous articular chondrocytes in vitro

We first investigated whether T cells from the OApatients reacted to autologous chondrocytes using a co-culture system. For this purpose, OA and normal chondrocytes were irradiated and then incubated with PBMC obtained from the same patients.

The results in Figure 1 show that PBMC from OA patients proliferated more strongly against the autologous chondrocytes than did normal controls.

Table I. Summary of the monoclonal antibodies used in the study

Specificity	Clone	Isotype	Source
HLAClass I (A, B and C)	W6/32	IgG2a	DAKO
HLAClass II (DP, DQ and DR)	CR3/43	IgG1a	DAKO
CD4	RPA-T4	IgG1	CSM
CD8	LT8	IgG1	CSM
HLA-DP	HI43	IgG1	PHARMINGEN
HLA-DQ	T_169	IgG2a	PHARMINGEN
HLA-DR	T_36	IgG1b	PHARMINGEN

SBA: Southern Biotechnology Association, Inc.; CSM: COSMO-BIO Co. Ltd, Tokyo Japan; R&D: R & D Systems, Inc.

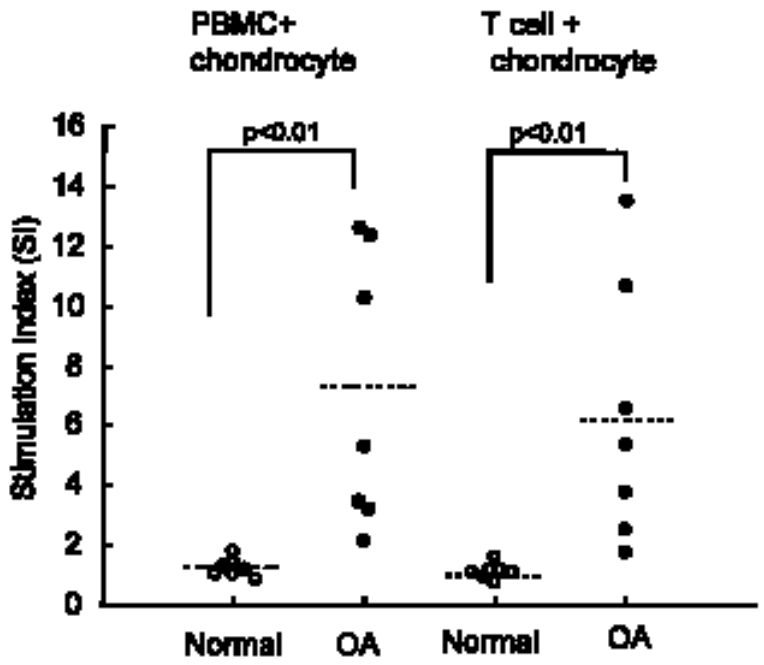


Fig. 1. Response of Tcells to autologous chondrocytes from OApatients or controls.
PBMC or T cells were co-cultured with autologous chondrocytes from OApatients (n = 7) and normal controls (n = 7). The proliferative responses were assayed by the uptake of ^{3}H thymidine and expressed as a stimulation index (SI) The SI values of both PBMC and T cell were higher in OA. Dotted lines indicate the mean values. The SI values were compared between normal and OA subjects using the Mann-Whitney U-test.

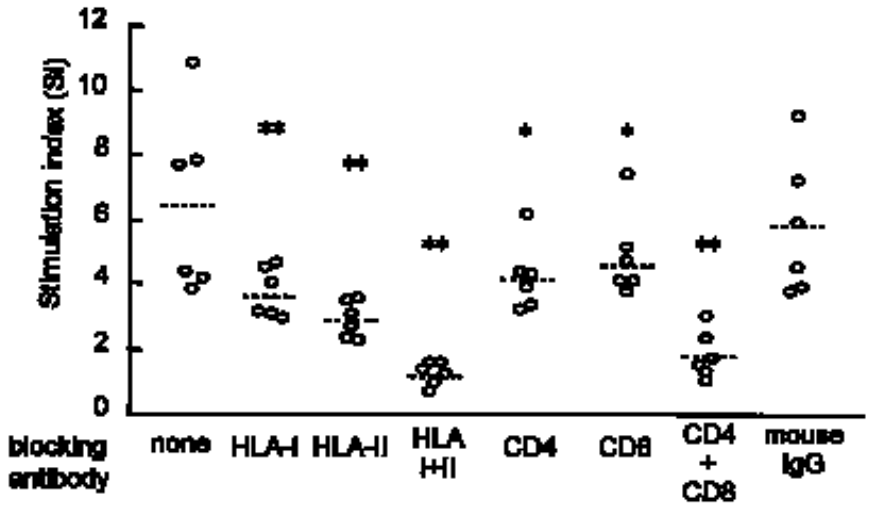


Fig. 2. Inhibition of T cell/chondrocyte interaction. Proliferative responses of PBMC to autologous chondrocytes were inhibited by various antibodies against cell surface molecules in OA (n = 6). The responses were blocked in part by each of the anti-HLAclass I, class II, CD4, and CD8 antibodies and blocked completely by the combinations of anti-HLAclass I and class II antibodies, and anti-CD4 and CD8 antibodies.
Dotted lines indicate the mean values. The SI values were compared between "none" and each antibody using the Mann-Whitoney U test. *p < 0.05, **p < 0.01.

The mean SI of PBMC was 7.34 ± 1.87 in OA, which was significantly higher than that in normals (1.28 ± 0.14, p < 0.01).

Because the PBMC contained monocytes which might act as APC, we isolated T cells from the PBMC population and again assessed their response to the chondrocytes. We found that the purified T cells from the OA patients also showed similarly significant responses to their autologous chondrocytes (SI 6.35 ± 1.63). This indicates that the response was not triggered by the autologous monocytes, but that the chondrocytes themselves do act as a stimulator for the autologous T cells. In contrast, PBMC and T cells from the trauma patients did not show proliferative responses against the autologous chondrocytes (SI 1.21 ± 0.09). Taken together, the T cells from the OA patients would respond to their own chondrocytes specifically.

Both CD4 and CD8 T cells respond to autologous chondrocytes

We next investigated whether the detected T cell response involved antigens presented by the MHC molecules. To address this issue, various monoclonal antibodies that block TCR/MHC binding were added to the co-cultures (Fig. 2). The SI of the OA derived PBMC co-cultured with autologous chondrocytes was 6.4 ± 1.1 (the SI of PBMC alone was 1.0). This response was shown to be blocked only slightly by the non-specific mouse IgG (5.1 ± 0.9). In contrast, the monoclonal antibodies against MHC class I, class II, or mixtures of these significantly inhibited the proliferative response of the T cells (2.5 ± 0.3, 2.4 ± 0.2, and 1.1 ± 0.1, respectively). These results suggest that both class I and class II-dependent responses were involved.

We further analyzed which populations of T cells were responsible for the response to the autologous chondrocytes. We found that both anti-CD4 and anti-CD8 antibodies inhibited the response significantly (2.6 ± 0.4, 3.1 ± 0.5, respectively). Furthermore, when both anti-CD4 and anti-CD8 antibodies were used in combination, the response of PBMC against chondrocytes was almost completely blocked (1.3 ± 0.3). Therefore, it is concluded that both CD4- and CD8-positive T cells are responsible for the HLA class I- and class II-dependent responses.

Expression of HLA class II molecules by chondrocytes

Since above we found significant T cell responses against the autologous chon-

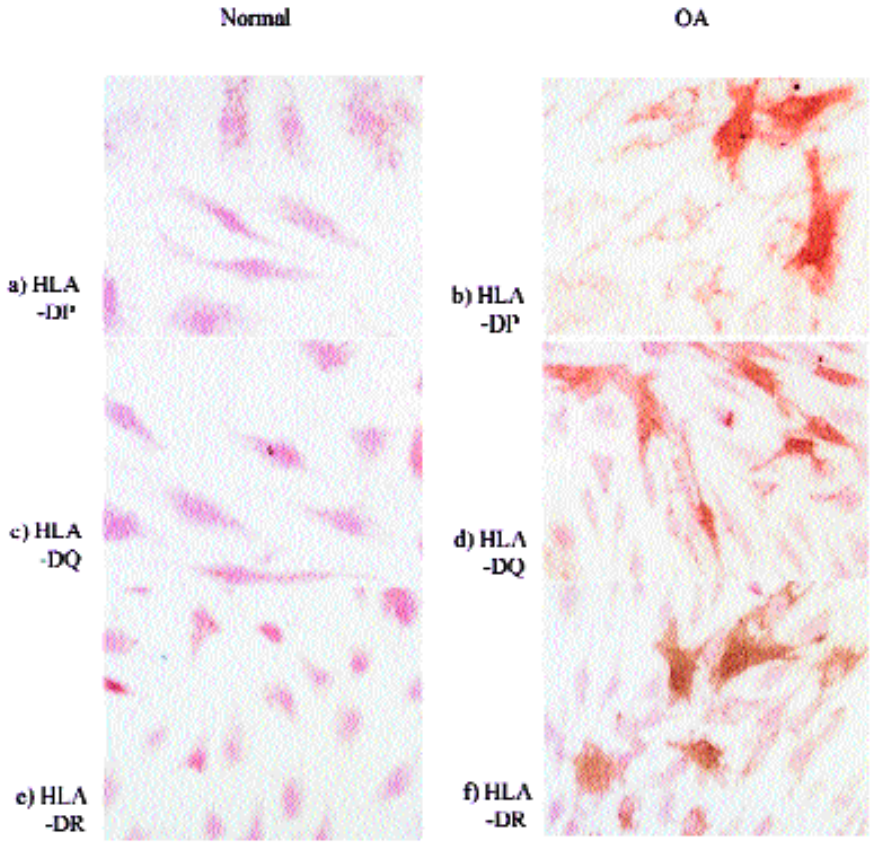


Fig. 3. Expression of HLA-DP, –DQ and –DR. Representative photomicrographs of normal (a, c, e) and OA(b, d, f) chondrocytes are shown. Staining of HLA-DP(a, b), HLA-DQ (c, d), and HLA-DR (e, f) is demonstrated. The magnification is x100.

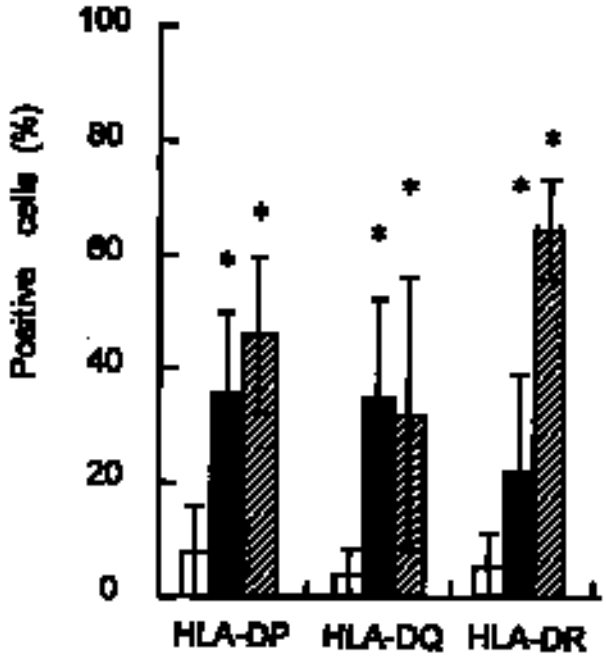


Fig. 4. Frequency of chondrocytes expressing HLA-DP, –DQ and –DR. The positive cells for each antibody in the immunocytochemical studies were counted and the percentages of OA and RA were compared to normal subjects using the Mann-Whitney U-test. *$p < 0.05$, **$p < 0.01$. The frequencies of OAand RAchondrocytes were higher than those of normal, whereas no significant difference was found between OAand RA, except for HLA-DR.

drocytes in a HLA-class I and class-II dependent manner, it was strongly implied that the chondrocytes present autoantigens on their MHC molecules. To confirm this fact, we investigated the expression of various MHC class II molecules by the chondrocytes.

The results of immunohistochemistry (Fig. 3) showed constitutive expression of the HLA-DP, DQ and DR antigens in OA patients (percentage of positive cells 36.1%, 34.9% and 22.4%, respectively). Although the percentages of positive cells in OA were apparently lower than in RA except for DQ (46.5%, 32.8% and 64.5%, respectively), they were significantly higher than the percentages in traumatic patients (13.9%, 17.7% and 16.3%, respectively).

Discussion

Our findings may be summarized as follows. Firstly, PBMC and in particular T cells showed proliferative responses against autologous chondrocytes in OA. Secondly, this response was blocked partially by one of the anti-HLAclass-I and class II antibodies and was blocked almost completely by the combined use of the two antibodies. Similarly, the response was partially blocked by one of the anti-CD4 and CD8 antibodies, and was blocked nearly completely by the combined use of the two antibodies. Finally, the OA chondrocytes express HLA-DQ, –DP, and –DR at higher percentages than in normal chondrocytes.

Since the proliferative responses of T cells against chondrocytes in OA were inhibited by anti-MHC antibodies, the MHC molecules were essential for the proliferation. Most likely, cognate interaction of T cells with chondrocytes via the MHC/antigen/TCR trimolecular complex would be an essential event. However, soluble factors derived from the OA chondrocytes may play additional roles which should be investigated in the future. In the case of MHC/antigen/TCR binding, the stimulating antigens would be produced by the chondrocytes. Previously the existence of a humoral immune response to membrane fractions of human chondrocytes was reported in RA and OA

patients (25), suggesting the presence of autoantigen(s) in the chondrocyte membranes. Furthermore, collagens and proteoglycans have also been reported as chondrocyte-derived autoantigens in OA. More recently we found that CILP, YKL-39 and osteopontin, which are mainly produced by chondrocytes, were recognized as autoantigens in patients with OA (9, 26). These proteins could be candidate antigens in the T cell response described here. In addition, T cells in the OA synovium display activated phenpotypes of CD69+, CD25+, CD45 RO+, and HLA-DR+ (4), and oligoclonal expansion, which indicate an antigen-specific immune response (5). These reports support the hypothesis of antigen-specific autoimmune responses in the OAjoints.

It is still unclear whether OA chondrocytes exert their effect as an efficient APC. Alsalameh *et al.* reported that chondrocytes from healthy individuals had a weak antigen presenting capacity, and could also process exogenous antigens (16). In addition, chondrocytes were reported to have the ability to phagocytose collagen fragments, hydroxyapatite and calcium pyrophosphate dihydrate particles (27, 28). Therefore normal chondrocytes would be potent APCs, even though they are not professional ones. Phenotypically, Burmester *et al*. reported that 30–87% of unstimulated OA chondrocytes expressed MHC class II molecules (29). More recently, Lance *et al*. reported that HLA-DR was detected in 3% of untreated chondrocytes in OA, 16% in RA and 2% in normal specimens (15). These observations support the idea that chondrocytes may be more potent APCs in some arthropathies. We here detected that OA chondrocytes expressed HLA-DQ, –DP, and –DR molecules at higher percentages than in normal chondrocytes. This also supports the hypothesis of a potent APC function of chondrocytes. With regard to this issue we are now exploring the expression of co-stimulatory molecules, CD80/CD86, on chondrocytes.

It is of interest that the T cell proliferation was partly inhibited by the anti-CD4 antibody alone and anti-CD8 antibody alone, but inhibited nearly completely by the mixed use of the two antibodies. This indicates that both of CD4+ and CD8+ T cells proliferated against the chondrocytes. Since activated CD4+ T cells play critical roles in producing proinflammatory cytokines like IL-1 and TNF-α in RA, the expanded CD4+ T cells may also play critical roles in cartilage degradation of OA. One of the possible roles of the reacted CD8+ T cells would be a cytotoxic effects on chondrocytes. Functional studies of the expanded T cells would be needed.

It remains to be solved why the autoreactive T cells against chondrocytes (or chondrocyte-producing proteins) are activated in OA and whether the autoimmunity to chondrocytes is pathogenic or not. On the former question, at least three factors should be pointed out. First, expression of cartilage-related proteins can be altered in OA. For example, CILP and several chondrocyte-related proteins were reported to increase their expression in the early stages of OA (30). Second, activated chondrocytes present the autoantigens to T cells. The up-regulated expression of HLAclass-II would reflect such activation. Thirdly, in the process of cartilage degradation in OA, cryptic epitopes in the cartilage-related proteins may be exposed to T cells. On the latter question, autoimmunity to the cartilage components or chondrocytes may cause mild but continuous damage to the cartilage which initiates degradation. Alternatively, during the process of the primarily non-inflammatory degradation of cartilage in OA, the degraded cartilage antigens may be presented to T cells, which enhance the degradation of cartilage immunologically. Finally, it cannot be excluded that the autoimmune response detected here represents an “epiphenomena” of OA.

In conclusion, we here demonstrated autoreactive T cells to chondrocytes and the high expression of HLA-DQ, DR, and –DP on chondrocytes in OA. This would imply possible pathophysiological roles of the autoimmune reaction involving T cells and chondrocytes in OA.

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