A novel paradigm for dendritic cells as effectors of cartilage destruction

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Objective. Dendritic cells (DCs) are enriched in RA synovium and have been implicated in the pathogenesis of RA primarily through their ability to present autoantigen and activate T cells. However, whether DCs play an effector role in cartilage destruction is unknown. The aim of this study was to investigate whether DCs can induce collagen release from cartilage and the mechanism involved.

Methods. Human monocyte-derived DCs (mDCs) were activated with CD40 ligand (CD40L) to mimic DC–T-cell interaction, and supernatants were incubated with cartilage explants. Hydroxyproline was assessed as a measure of collagen release and collagenolytic activity was measured by a bioassay using tritiated collagen. TNF-α in DC supernatants was measured by specific ELISA.

Results. Supernatants from CD40L-activated mDCs, but not unstimulated mDCs, strongly induced the destruction of cartilage collagen. mDC supernatants did not contain collagenases but did induce collagenolytic activity in cartilage explants. Neutralization of TNF-α in mDC supernatants completely abolished collagenolysis.

Conclusions. This study shows that mDCs, upon CD40-ligation, induce cartilage collagen degradation through an indirect mechanism via the production of TNF-α. Our data suggest a potential important role for mDC-derived TNF-α in RA, which is in line with the previously reported observations that DCs are a major source of TNF-α in early autoimmune lesions and that anti-TNF-α therapeutics effectively suppress joint damage in RA patients. We propose that DCs can act as effectors in cartilage destruction, adding a new aspect to the functional role of DCs in RA pathogenesis.

Key words: Dendritic cells, Cartilage destruction, Collagen resorption, Tumour necrosis factor-α.

Introduction

RA is the most common inflammatory joint disease, affecting ~1% of adults [1]. It is a progressive and debilitating illness characterized by chronic synovial inflammation, causing destruction of cartilage and bone. Although the aetiology of RA is unknown it is generally accepted that RA is an autoimmune disease in which various cells of the immune system play a role, including dendritic cells (DCs) [2, 3]. DCs are potent antigen-presenting cells that are pivotal for the initiation and regulation of immune responses [4]. DCs are strategically located at potential sites of pathogen entry, such as peripheral epithelial and mucosal tissues. Upon encountering a pathogen, DCs undergo maturation as defined by up-regulation of MHC Class II (MHCII) molecules and co-stimulatory molecules (e.g. CD86) and migrate to lymph nodes, where they activate antigen-specific T cells.

DCs have also been demonstrated to be present in the SF and synovial tissue of RA patients [5–7], and it has been proposed that DCs are involved in the pathogenesis of RA in several ways [2, 3]. Primarily, DCs can drive pathogenic autoimmune responses by presenting self-antigens to T cells. In experimental models, it has been shown that arthritis and other autoimmune diseases can be triggered by DCs presenting self-antigen [8–10]. Furthermore, the synovial tissue of RA patients is enriched with DCs with high T-cell stimulatory capacity as compared with normal synovium [7, 11, 12]. Rheumatoid synovial DCs are often surrounded by T cells, and it is thought that these DCs present arthriticogenic antigens to T cells, thereby perpetuating the inflammatory immune response [2, 3]. Secondly, it is likely that DCs contribute to the formation of lymphoid tissue in the rheumatoid synovium. DCs are a source of factors that promote lymphoid neogenesis, such as TNF family members and various homing chemokines for T cells, B cells and macrophages [3, 10, 13], and it has been shown that DCs drive ectopic lymphoid tissue formation in autoimmune diabetes model [8]. Finally, there is accumulating evidence that DCs are also involved in atherosclerosis [14], a serious complication of RA. DCs can be found in atherosclerotic plaques, where they co-localize with T cells and contribute to arterial inflammation and acceleration of atherosclerosis [14, 15].

Here, the involvement of DCs in another aspect of RA was studied: the destruction of cartilage. One of the hallmarks of RA is the excess proteolysis of the articular cartilage extracellular matrix, in which type II collagen and aggregan are the two major structural components. Aggreican can be rapidly released from cartilage in response to a number of pro-inflammatory cytokines, but it can also be replaced relatively quickly [16]. In contrast, the degradation of collagen is less rapid, but is irreversible and leads to permanent structural damage to cartilage tissue [17]. The breakdown of collagen is therefore considered to be a key event in RA and other arthritic diseases. In healthy cartilage, collagen homeostasis is maintained by balancing the production and activity of collagenases (e.g. MMP-1, -8 and -13) and tissue inhibitors of metalloproteinases (TIMPs). Expression of these molecules is regulated by a variety of cytokines and growth factors and dysregulation leads to uncontrolled collagenolysis [18, 19].

In this study, the question of whether DCs can drive cartilage collagen destruction was addressed. Monocyte-derived DCs (mDCs) were used, because they are a good in vitro model for the type of myeloid DCs present in inflamed tissues such as the rheumatoid synovium [20, 21]. The ability of these mDCs to destroy cartilage collagen and the underlying mechanism was investigated.

Materials and methods

Ethics

Human samples were obtained with informed consent according to the Declaration of Helsinki and the study was approved by the North Tyneside Research Ethics Committee.
Cartilage destruction by dendritic cells

Culture media, cytokines and TNF-α antagonists

RPMI-1640, DMEM, penicillin, streptomycin and nystatin were purchased from Sigma (Poole, UK). Fetal bovine serum (FBS) was from PAA laboratories (Pasching, Austria). Recombinant human IL-4 and GM-CSF were obtained from Immunotools (Friesoythe, Germany). Recombinant human oncostatin M (OSM) was kindly provided by Professor John Heath (Birmingham University, Birmingham, UK) and recombinant human IL-1α was a generous gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, UK). The therapeutic anti-TNF-α monoclonal antibody infliximab was from Centocor BV, Leiden, The Netherlands and the soluble human TNF-receptor p55 Ig fusion protein (TNFR-Ig) was from the Oxford Therapeutic Antibody Centre, Oxford, UK [22]. Campath-1H (alemtuzumab; therapeutic anti-CD52 monoclonal antibody) was used as a control.

Monocyte-derived DCs

Human mDCs were generated using a well-established in vitro culture protocol [23–25]. Briefly, peripheral blood mononuclear cells were isolated from peripheral blood by density centrifugation on Lymphoprep (Axis-Shield Diagnostics, Dundee, UK). CD14+ monocytes were isolated by positive selection using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate mDCs, monocytes were cultured at 0.5 × 10^6 cells/ml in the presence of IL-4 and GM-CSF (50 ng/ml each), in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 C with 5% CO₂. After 6 days, non-adherent mDCs were harvested and macrophage contamination was excluded by staining for CD14 and flow cytometry. mDC-conditioned media were prepared as follows: mDCs were extensively washed to remove cytokines and FBS and replated at 0.25 × 10^6 cells/ml in the absence or presence of CD40-ligand transfected J558L mouse cells (kindly provided by Professor Peter Lane, Birmingham University, Birmingham, UK) at a 1 : 1 ratio in serum-free DMEM supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and nystatin (20 IU/ml). After 24 h, cell-free supernatants were harvested and stored at −80°C, and cells were harvested for surface marker analysis by flow cytometry. Human TNF-α was quantified using a specific sandwich ELISA (BD Pharmingen, San Jose, CA, USA).

Flow cytometry

The following antibodies were used: anti-CD1a-FITC (NA1/34; Dako, Glostrup, Denmark); anti-CD14-PE (M5E2); anti-CD80-PE (L307.4); anti-CD86-FITC (2331); anti-HLA-DR-APC (L243; all from BD Pharmingen). Isotype-matched control antibodies were used to confirm that staining was specific (data not shown). Cells were centrifuged and resuspended in FACS buffer (phosphate-buffered saline supplemented with 3% FCS, 2 mM EDTA and 0.01% sodium azide). Human IgG (Grifols, Los Angeles, CA, USA), was added with antibodies to prevent Fc receptor binding. Cells were incubated on ice for 30 min, washed and resuspended in FACS buffer. Data were collected on a Becton Dickinson FACScan and analysed using FlowJo (Treestar, Ashland, Oregon, USA).

Cartilage degradation assay

A well-established bovine nasal cartilage explant degradation assay was used, which has been validated as a relevant model for studying cartilage collagen destruction in human disease [26–28] and has been used extensively to study the effects of human regulators of cartilage destruction e.g. human cytokines, MMPs and TIMPs [26–31]. Discs (2 mm³) were punched from bovine nasal septum cartilage. Three discs per well in a 24-well plate were cultured in 600 μl control medium: serum-free DMEM supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and nystatin (20 IU/ml). After 24 h, the control medium was replaced with fresh control medium (negative control), control medium with OSM (10 ng/ml) and IL-1 (1 ng/ml; positive control) or mDC-conditioned medium. TNF-α antagonists or control antibodies were added as indicated. Four wells for each condition were cultured for 7 days at 37°C. Supernatants were harvested and the cartilage explant cultures were replenished with identical treatments as on Day 1 and cultured for an additional 7 days and again supernatants were harvested. Day 7 and day 14 supernatants were stored at −20°C for further analysis. The remaining cartilage discs were digested with papain (4.5 mg/ml; Sigma) in 0.1 M phosphate buffer, pH 6.5, containing 5 mM EDTA and 5 mM cysteine hydrochloride, for 16 h at 65°C. Digests were stored at −20°C until further analysis.

Collagen release and collagenolytic activity assays

Cartilage explant culture supernatants and cartilage digests were assayed for hydroxyproline (OHPro) as a measure of collagen using modifications of the OHPro assay as previously described [26]. The release of collagen was calculated as a percentage of the total.

Collagenolytic activities present in mDC-conditioned media and in supernatants from cartilage explants were determined by a 96-well plate modification of the diffuse fibril assay using [3H]-acylated collagen [32]. Pro-collagenases were artificially activated by co-incubation with 0.7 mM 4-aminophenylmercuric acetate (APMA; Sigma) in order to obtain total (pro- and active) collagenolytic activity. One unit of collagenase activity degrades 1 μg of collagen per minute at 37°C.

Statistics

The unpaired t-test was performed using Prism 4.00 (GraphPad Software, San Diego, CA, USA). All P-values are two-tailed.

Results

mDCs up-regulate surface markers in response to CD40 ligation

To confirm that co-culturing of mDCs and CD40L-expressing cells resulted in mDC activation as shown previously by us and others [33, 34], the expression of cell surface molecules by mDCs was analysed (Fig. 1). This is a well-established system and only CD40L-transfected but not non-transfected J558L cells have the ability to induce up-regulation of surface markers and production of cytokines by DCs [33]. Typically, both unstimulated and CD40L-activated DCs express the DC marker CD1a but lack expression of the macrophage/monocyte marker CD14. As expected, activation of mDCs through CD40 ligation did enhance the surface expression of classical molecules involved in T-cell activation, i.e. the antigen-presenting molecule HLA-DR and the co-stimulatory molecules CD80 and CD86.

CD40L-activated mDCs induce cartilage collagen destruction

To determine whether CD40L-activated mDCs were able to promote cartilage resorption, conditioned media from unstimulated mDCs, CD40L-expressing J558L cells and CD40L-activated mDCs were incubated with cartilage explants for 14 days and the release of collagen fragments was measured (Fig. 2). The combination of IL-1 + OSM was used as a positive control; our group has shown previously that these cytokines synergistically promote collagen degradation from cartilage in vitro and in vivo [26, 35]. Conditioned medium from unstimulated mDCs and CD40L cells did not induce collagen release. However, conditioned medium from CD40L-activated DCs strongly induced collagen resorption. Thus, activation of mDCs through CD40 ligation induces the production of soluble factor(s) that have the ability to lead to collagen and cartilage destruction.

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CD40L-activated mDCs destroy collagen through an indirect mechanism

We next addressed the question of whether the destruction of collagen by CD40L-activated mDCs was through a direct (e.g. secretion of collagenases) or indirect (e.g. production of cytokines that induce collagenase production by chondrocytes in cartilage) mechanism. The amount of active and total collagenolytic activity was determined in conditioned media before and after incubation with cartilage (Fig. 3). As expected, supernatants of unstimulated mDCs and CD40L-expressing cells did not express any collagenolytic activity before or after co-culture with cartilage, whereas supernatants from IL-1 + OSM-treated cartilage explants expressed high collagenolytic activity. Conditioned medium from CD40L-activated DCs only exhibited the ability to lyse collagen after they had been incubated with cartilage. The lack of collagenolytic activity in CD40L-activated mDC supernatants before and after culture with the cartilage explants. As a positive control supernatants of IL-1 + OSM-treated cartilage explants were used. Results are expressed as collagenolytic units, one unit representing degradation of 1 μg of collagen per minute at 37°C. Results are expressed as mean ± S.D. of quadruplicate cultures and are representative of two independent experiments. **P < 0.001; *P < 0.0001.
failed to produce any collagenases upon activation through CD40 and thus did not exert any direct collagenolytic activity, mDCs promoted cartilage collagenolysis indirectly through TNF-α production.

A variety of molecules can activate DCs, including Toll-like receptor (TLR) ligands (e.g. lipopolysaccharides) and pro-inflammatory cytokines (e.g. IL-1 and TNF-α) as well as CD40L, which is expressed by activated T cells. In this study, the latter activation signal was chosen, because (i) CD40L activation of DCs is a physiologically relevant process in synovitis [37] and (ii) it is not possible to use TLR ligands or pro-inflammatory cytokines for studying cartilage destruction by activated DCs because these signals can directly promote such destruction [38, 39]. CD40–CD40L interactions play an important role in immune regulation [40]. Cross-talk between DCs and T cells through CD40-CD40L enhances the T-cell stimulatory capacity of DCs by up-regulating the expression of co-stimulatory molecules (e.g. CD80 and CD86) and the production of cytokines important for T-cell activation and differentiation (e.g. IL-12). In RA patients, synovial DCs are often surrounded by T cells [20] and synovial T cells express enhanced levels of CD40L [41, 42], facilitating synovial DC activation through CD40 ligation. Increased expression of CD40L on T cells in RA patients has been shown to be associated with active disease [42]. It has been proposed that CD40L aggravates rheumatoid synovitis by enhancing the production of pro-inflammatory cytokines in synovial cells [43, 44] and by augmenting the production of macrophage migration inhibitory factor (MIF) in DCs from RA patients [45].

Our data show that activation of mDCs through CD40L results in the production of catabolic soluble mediator(s) that promote cartilage collagenolysis via the production of collagenolytic MMPs in cartilage tissue. TNF-α was found to be a major destructive mediator in mDC supernatants since TNF-α neutralization abolished collagen resorption. We have shown previously that rTNF-α strongly promotes collagen breakdown on its own, but also synergizes with other cytokines [27, 28, 46]. For example, the combination of TNF-α with either OSM or IL-1 induces a significantly higher expression of MMP-1 and -13 expression in chondrocytes and leads to the synergistic release of collagen from cartilage [28, 46]. CD40L-activated mDCs did not secrete any detectable levels of OSM but did produce IL-1β (IL-1β was derived from human mDCs and not from murine J558L cells as no detectable levels of murine IL-1β could be detected by ELISA; data not shown). Furthermore, mDCs produce high levels of IL-6 and the soluble IL-6R (sIL-6R) upon CD40 ligation ([25, 47] and data not shown) and these cytokines have also been shown to synergize with other soluble mediators in the destruction of cartilage collagen [48]. Thus, although mDC-derived TNF-α is a major player in driving cartilage breakdown, synergism with other cytokines such as IL-1 or IL-6/sIL-6R cannot be excluded. Nevertheless, exclusion of mDC-derived TNF-α effectively abolished the observed cartilage destruction. This potent inhibitory effect of anti-TNF-α may in part explain why therapies targeting TNF-α are so successful in suppressing joint damage in RA patients [49, 50].

The high number of DCs required to perform the experiments described here precluded such studies with DCs isolated from synovial tissue or SF. However, we used mDCs as a model for the type of myeloid DCs found in the rheumatoid synovium because monocytes are precursors of myeloid inflammatory DCs in chronic inflammatory conditions such as arthritis [20, 21, 51]. Furthermore, mDCs resemble myeloid DCs derived from RA SF phenotypically (e.g. expression of CD1a, CD11c, HMCII, CD80, CD86 and CD40) and functionally (e.g. the potent ability to stimulate T cells and to drive Th1 inflammatory-type responses) [52, 53].

In summary, DCs have been implicated in both the initiation and perpetuation of RA. Because DCs are potent antigen-presenting cells, a currently accepted view is that their main role in the disease process is to present autoantigens to T cells, thereby
driving the inflammatory autoimmune response. Here, we have demonstrated that DCs are also capable of inducing the release of cartilage collagen, a hallmark of joint destruction. Our data add a new aspect to DC function in RA. In addition to their well-known immunoregulatory role our data suggest that they also play a role as effector cells in joint destruction. This novel role of DCs in the pathogenesis of RA fits with previous in vivo observations that DCs are amongst the first immune cells to be recruited into inflammatory tissues at early stages of autoimmune diseases such as diabetes and arthritis, and together with macrophages are a major source of TNF-α [51, 54]. Targeting synovial DCs in early RA may therefore be an attractive therapeutic strategy, since it would not only interfere with the presentation of articular autoantigens to T cells but also with the effector function of DCs in cartilage breakdown. Such early, targeted interventions could markedly alter disease pathogenesis with significant long-term benefits.

**Rheumatology key messages**

- Myeloid DCs can act as effector cells in cartilage destruction.
- TNF-α plays a key role in collagen breakdown by myeloid DCs.

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