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Disease status in human and experimental arthritis, and response to TNF blockade, is associated with MHC class II invariant chain (CD74) isoform expression

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A B S T R A C T

Splice variants of CD74 differentially modulate the activity of cathepsin L (CTSL). As CD74 and CTSL participate in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA), we determined whether splice variants of CD74 could be biomarkers of disease activity. Gene expression was measured in mice with collagen-induced arthritis using quantitative PCR (qPCR). In vitro studies using murine macrophage/DC-lineage cells determined the relative influence of macrophage phenotype on isoform expression and the potential to produce CTSL in response to TNF. CD74 splice variants were measured in human RA synovium and RA patients' monocytes. In arthritic mice, the expression of the p41 CD74 isoform was significantly higher in severely affected paws compared with unaffected paws or the paws of naïve mice; the p41 isoform significantly correlated with the expression of TNF in arthritic paws. Compared with M2-like macrophages, M1-like macrophages expressed increased levels of CD74 and had higher expression, secretion and activity of CTSL. RA patients that responded to TNF blockade had significantly higher expression levels of CD74 in circulating monocytes after treatment, compared with non-responders. The expression of the human CD74 isoform a was significantly higher in RA synovia, compared with osteoarthritis synovia, and was associated with CSTL enzymatic activity. This study is the first to demonstrate differential expression of the CD74 p41 isoform in an auto-immune disorder and in response to therapy. The differential expression of CD74 splice variants indicates an association, and potentially a mechanistic role, in the pathogenesis of RA.

1. Introduction

The MHC class II invariant chain (also known as Ii and, at the cell surface, CD74), is required for the efficient assembly and trafficking of MHC-II α:β chain heterodimers to the endosome wherein antigen loading occurs [1]. The classical role of CD74 is as a scaffold in antigen presentation, although it is now recognised that this molecule participates in several immune-related processes including B-cell maturation, dendritic cell (DC) motility and as a receptor for macrophage migration inhibitory factor (MIF) [2]. Due to RNA splicing, CD74 is present in several isoforms expressed at different ratios depending on cell type, with expression of the larger variant (termed p41 or 'isoform 1' in mice and 'isoform a' in humans) being higher in macrophages (Mφ) and DC than in B-cells [2], but always lower than the smaller p31 variant regardless of cell type. The p41 isoform and its human equivalent contain a type I thyroglobulin-like domain which binds to and modulates the catalytic activity of cathepsin L (CTSL) [3,4]. In addition to homologs of the murine variants, humans have a further 2 forms of CD74 which have minor differences to the two main homologs due to an alternative translation start site [5].

Cathepsins participate in several physiological and pathophysiological processes, such as angiogenesis, cell migration and intracellular degradation of antigens [6]. CTSL is one of several proteases that participates in the degradation of CD74 during the process of antigen loading and can thereby influence antigen-specific immunity [1,3,4]. CTSL is synthesized as an inactive pro-enzyme that converts to the active form due to decreasing pH in the maturing endocytic vesicle. However, by binding to the thyroglobulin-like domain in the CD74 p41

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isoform, the activity of CTSL within the endosome is reduced [7]. In addition to resisting CTSL digestion, the thyroglobulin-like domain in the p41 isoform has been shown to stabilize CTSL at a neutral pH, which permits the accumulation of the complex in the extracellular space [8]. Cathepsin B, K and S are also produced at sites of active disease in patients with rheumatoid arthritis (RA) and in experimental arthritis, and mediate cartilage and/or bone destruction [9]. As well as antigen presentation and CTSL secretion, a third consequence of the relationship between CD74 and CTSL on immune cell function is the influence on cell motility, as the intracellular N terminus of CD74 binds to and sequesters myosin II [10]. CD74 remains bound to myosin II until digested by CTSL; once freed, myosin II is able to facilitate migration by altering the cytoskeleton [10].

Depending on the maturity or activation status of the cell, the relative abundance of CTSL and isoforms of CD74 may thus determine the cellular response to inflammation. Despite the participation of cathepsins in disease processes, and the interaction of CD74 and CTSL, the expression of CD74 isoforms in arthritis has not been evaluated in great detail. Most studies of CD74 in arthritis have instead focussed on its role as a receptor for MIF [11–14] or the effect of cathepsin inhibition [15]. As the p41 variant can modulate cell motility, antigen presentation and CTSL secretion, and these processes are modulated in disease, our aim was to determine to what degree CD74 splice variants were associated with inflammatory disease status.

In order to characterise the relationships between CD74 splice variants and disease activity, differential expression of the two principal CD74 splice variants, p31 and p41, was measured in the lymph-nodes, spleen and paws of mice with collagen-induced arthritis (CIA); expression at the gene and protein levels was also measured in the Mφ/DC-lineage cells known to be present in these tissues, by generating differentially polarised Mφ, DC and osteoclasts (OCL) *in vitro* as well as human monocyte-derived macrophages primed with a range of inflammatory mediators. The effects of an inflammatory stimulus on the expression of CD74 splice variants in M1-and M2-like Mφ were determined, as well as the resulting production and activity of CTSL. In RA patients, gene expression levels of CD74 isoforms were measured at the site of active disease, the synovium, as well as in blood monocytes before and after treatment with TNF blockade.

2. Material and methods

2.1. Mice

All procedures were conducted in accordance with UK Home Office guidelines and regulations. DBA/1 mice were immunized with bovine type II collagen emulsified with complete Freud's adjuvant as previously described [16]. Arthritis severity was assessed as follows: 0, normal; 0.5, slight swelling and/or erythema; 1, clear swelling; 2, pronounced oedematous swelling. Ten days after the onset of the disease, the animals were euthanised and spleen, inguinal lymph-nodes and paws harvested.

2.2. RNA extraction

Mice paws were snap-frozen in liquid nitrogen and pulverized with the BioPulverizer™ (BioSpec). Pulverized paws were homogenised in 500 μL of TRIzol reagent (Invitrogen) using the Sample Grinding Kit (GE Healthcare). The aqueous phase of the phenol/chloroform extraction was mixed with an equal volume of 70% ethanol and added to an RNA isolation column (RNeasy Mini Kit, Qiagen) and RNA extracted according to manufacturer's instructions. Spleens and lymph nodes were dissociated through a cell strainer, washed and RNA extracted using Trizol; RNA from *in vitro*-differentiated cells was also extracted using Trizol.

2.3. Bone marrow-derived macrophage-lineage cells

Bone marrow (BM) cells from naïve mice were cultured in medium (10% FBS, 1% penicillin/streptomycin in RPMI1640 with L-glutamine (Invitrogen)), with the addition of the following cytokines: GM-CSF 50 ng/mL (for M1-like Mφ), M-CSF 25 ng/mL (M2-like Mφ), GM-CSF 50 ng/mL and IL-4 10 ng/mL (DC) and M-CSF 25 ng/mL and RANKL 50 ng/mL (OCL); cells were seeded (2×10^6/well for Mφ and DC, and 8×10^6/well for OCL) and differentiated in these conditions for 7 days. In some experiments M-Mφ and GM-Mφ were stimulated with TNF 50 ng/mL. Murine cytokines were supplied by Peprotech with the exception of M-CSF (R&D Systems).

2.4. Human tissues

Ethical approval was obtained from the Riverside Research Ethics Committee, the City and East London Ethics Committee (06/Q0605/8; NRES Committee), the Ethics Committee of the Ministry of Health of Hungary and Ethics Committee of the University of Szeged. Tissues and blood samples were obtained from the Nuffield Orthopaedic Centre (Oxford) and The Royal Free London NHS Foundation Trust (London), Northwick Park Hospital (London), Imperial College Healthcare Trust (London) hospitals and the Albert Szent-Gyorgyi Health Centre (Szeged) after receiving informed consent.

2.5. Human synovia

RA and osteoarthritic (OA) synovial membrane cells were isolated from patients undergoing joint replacement surgery as previously described [17]. Gene expression was measured using a linearised plasmid containing genes of interest as a quantitative standard curve of 10-fold dilutions starting at 10^7 copies/rxn for qPCR as previously described [16,17]. In some experiments, synovial explants were cultured for 20 h in RPMI1640 containing 1% Buminate (5% human albumin supplement (Baxter Healthcare)) after which the conditioned medium was collected, filtered and stored at −80 °C for analysis. Where disclosed, the treatments for RA patients included Sulfasalazine (7%), Azathioprine (7%), analgesics (13%), Prednisone (33%), Leflunomide (20%), biologics (20%), and Methotrexate (47%).

2.6. Human blood monocytes

Blood was collected in EDTA tubes from RA patients prescribed anti-TNF treatment, before and 3 months after therapy; healthy controls samples were obtained from the NHS Blood Service. Poor response to therapy was defined as a reduction in DAS28 of less than 2.2; for several participants the collection at 3 m was not possible however Responder/Non-Responder status was determined for all patients. Peripheral blood mononuclear cells (PBMCs) were enriched on density gradients and then washed extensively to remove platelets. Monocytes were isolated from the PBMCs immuno-magnetically by CD14 positive selection (Miltenyi). RNA was extracted using an Isolate II RNA/DNA/Protein kit (Bioline). The purity of monocytes was determined by flow cytometry using antibodies for CD14 (HCD14, BioLegend Ltd); see Supplementary Table 1 for patient details. Monocyte-derived macrophages (MDM) were differentiated from healthy control monocytes with 5 days of culture in 10% FBS RPMI (10^7 cells/10mL/10 cm dish) supplemented with 50 ng/mL M-CSF (Peprotech); MDM were detached and re-plated into 12 well plates, allowed to attach for 24 h, then stimulated with LPS (10 ng/mL, Merck), LPS + IFNγ (10 ng/mL+10 ng/mL(Peprotech)), IL-4 (50 ng/mL, Peprotech), GM-CSF (50 ng/mL, Peprotech), TNF (50 ng/mL, Peprotech), GW1929 (1 μM, Enzo Life Sciences), TGF-β1 (100 ng/mL, Peprotech), VEGFA (100 ng/mL, Peprotech), IL-10 (50 ng/mL, Peprotech) or dexamethasone (DEX, 5 nM, Merck) for 20 h.

2.7. RT-PCR

Reverse transcription of 500 ng of RNA was performed (High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)) in a 40 μL reaction which was then diluted to a total of 120 μL in nuclease-free water. Expression of target genes was determined using TaqMan gene expression assays (Applied Biosystems) and expressed relative to HPRT gene expression using the ΔΔCT approximation method, except for human synovial samples and Mφ where the standard curve method was used (see Human synovia). The Taqman assays used for murine samples included total *Cd74* (Mm00658576_m1), *Cd74*-p31 (Mm01262766_m1), *Cd74*-p41 (Mm01266672_m1), *Tnf* (Mm99999068_m1), *Ctsl* (Mm00515597_m1) and *Hprt* (Mm00446968_m1). For human samples, gene expression was measured with the following Taqman assays *CD74* all isoforms (Hs00269961_m1), *CD74* isoform a (Hs00959496_m1), *CTSL* (Hs00964650_m1) and *HPRT1* (Hs99999909_m1).

2.8. ELISA

CTSL secreted into the cell culture medium was measured by direct ELISA. Using 96-well EIA plates (Costar), conditioned medium was added at a 1:9 dilution in coating buffer (100 mM Na_2CaO_3/$NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Recombinant murine Ctsl (R&D Systems) was used for constructing the standard curve. The plate was washed with PBS:Tween 20 (0.05% v/v) and blocked with 1% BSA for 2 h. After washing, the plate was incubated with polyclonal goat anti-murine CTSL antibody (1 μg/mL, R&D Systems) overnight at 4 °C. After washing, bound CTSL/CTSL antibody complex was detected with HRP-conjugated anti-goat for 4 h at RT. After washing, tetramethylbenzidine substrate was added and reaction stopped by the addition of H_2SO_4 and the absorbance at 540 nm measured using a Thermo LabSystems Multiscan Ascent plate reader.

2.9. CTSL activity assay

The enzymatic activity of secreted CTSL was determined using InnoZyme CTSL Activity kit (Calbiochem) according to the manufacturer's instructions. Fluorescence was measured on a FLUOstar Omega fluorometer (BMG Labtech) according to the manufacturer's instructions.

2.10. Western blotting

Proteins were isolated from the phenol/chloroform phase after RNA extraction. Samples (10 μg) were boiled in SDS loading buffer for 10 min and analysed by SDS-PAGE using a NuPage 10% Bis-Tris gel (Invitrogen). Proteins were transferred to a nitrocellulose membrane and the membrane blocked with 5% skim milk powder/PBS Tween20 (0.05% v/v). After washing, the membrane was incubated with purified rat anti-murine CD74 antibody (In-1, BD Pharmingen, at 1:1000 dilution), followed after washing by HRP-conjugated rabbit anti-rat antibody (polyclonal H&L $F(ab')_2$; Abcam, at 1:1000) to detect CD74 isoforms. β-actin was detected with monoclonal murine anti-β-actin (AC-15, Sigma; at 1:2000).

2.11. Statistical analyses

Data analyses were performed using GraphPad Prism software. Statistically significant differences determined using 1-way ANOVA or Student's t-test as appropriate. The Pearson correlation coefficient was used to determine correlations.

3. Results

3.1. The p41 splice variant is associated with disease activity in experimental arthritis

To map the expression of CD74 in disease, gene expression of total CD74 (Fig. 1A) and the p31 (Fig. 1B) and p41 (Fig. 1C) splice variants was measured by qPCR in spleens, lymph nodes and paws of naïve mice and mice with CIA. To determine the relative difference of the alternative splice variant, we determined the ratio of p41:p31 in each sample (Fig. 1D). Splenic expression of total CD74 tended to be higher in arthritic mice although the p31 splice variant was moderately lower in the spleen of arthritic mice compared with naïve mice. The expression of total CD74 was significantly higher in lymph nodes from arthritic mice compared with naïve mice (Fig. 1A), and this increase was attributable to a proportional increase in the p41 splice variant (Fig. 1C). In order to assess CD74 expression at the site of disease activity, paws from arthritic mice were divided into non-affected (UN, clinical score of 0) and severely affected (AFF, clinical score of 2) joints. Expression of total CD74 was significantly increased in paws with severe disease when compared with paws from naïve mice (Fig. 1A). Expression of the p31 splice variant was moderately increased in paws from arthritic mice. However, the increase in total CD74 expression appears to be mainly due to increased expression of the p41 splice variant, which was significantly higher in severely affected paws compared with naïve and also unaffected paws (Fig. 1C). The ratio of p41:p31 expression was significantly greater in the spleen of mice with CIA compared with naïve mice. The trend for greater relative p41 transcript expression was also reflected in the p41:p31 ratio in affected paws from arthritic mice (Fig. 1D).

3.2. Modulation of splice variant expression by TNF

As the expression of the p41 splice variant was higher in arthritic paws, to further demonstrate the association of each splice variant with inflammation we measured the expression of TNF, which is a key driver of disease [18]. There was a significant positive correlation between TNF gene expression and expression of the splice variants of CD74 in arthritic paws (Supplementary Fig. 1A and B (i-iii)); there was also a trend towards correlation between TNF and CD74 isoform expression in unaffected paws but no correlation in paws from naïve mice.

In arthritic mice, DC/Mφ-lineage cells (Mφ, DC and OCL) are active participants in multiple aspects of disease pathogenesis, including antigen presentation, pro-inflammatory cytokine production and bone resorption. In human RA the number of Mφ, in particular, correlates with the severity of lesions [19]. After differentiating M–CSF– and GM–CSF–derived Mφ, DC and OCL from bone marrow precursors *in vitro*, the relative expression of CD74 splice variants was determined in the differentiated cells. Significant differences in the gene expression of each transcript variant were detected (Supplementary Figure 2A). However, the p31:p41 expression ratio remained constant for the cells analysed. Stimulation with TNF for 2 or 20 h induced virtually no increase in gene expression in M-Mφ and highest expression by GM-Mφ at 2 h, but the difference in expression between these polarised macrophages remained significantly different (Supplementary Figure 2B). Differences in gene expression were also confirmed at the protein level (Supplementary Figure 2C). The failure to increase CD74 expression in stimulated M-Mφ was further demonstrated in human MDM differentiated in M-CSF. Cells were stimulated with medium alone, LPS, LPS/IFNγ, IL-4, GM-CSF, TNF, GW1929 (PPARγ agonist), TGF-β1, VEGFA, IL-10 and dexamethasone for 20 h (Supplementary Figure 2D). However, inflammatory stimuli were not capable of increasing expression of CD74 and several stimuli (TGF-β1, VEGFA, IL-10 and dexamethasone) appeared to further suppress CD74 expression.

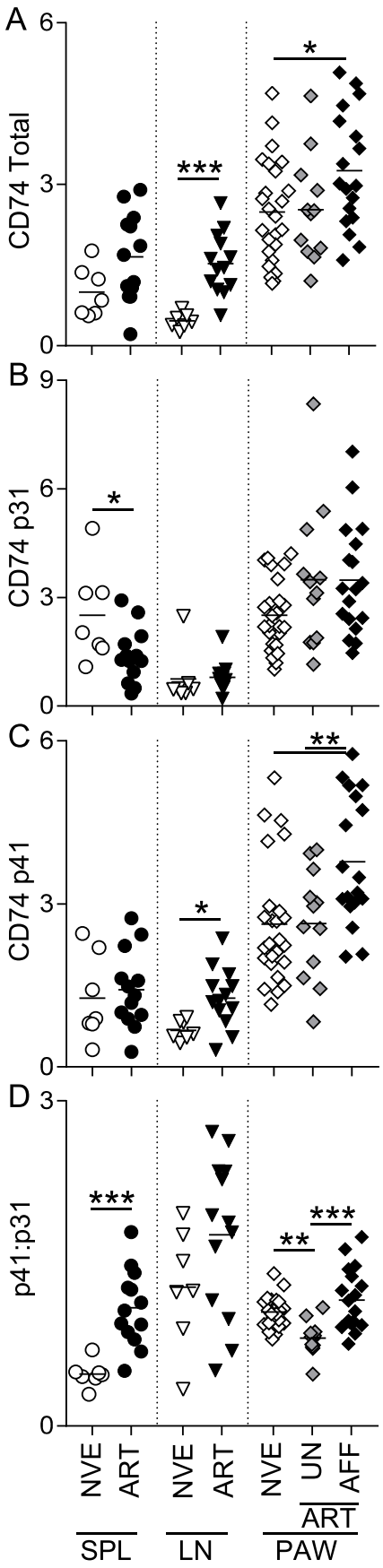

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Fig. 1. Expression of CD74 isoforms in naïve and arthritic mice. Gene expression of (A) total CD74 and of the two different isoforms (B) CD74 p31 and (C) CD74 p41 was measured and the ratio of (D) p41:p31 gene expression was calculated in spleens (SPL), lymph nodes (LN) and paws (PAW) of naïve (NVE) and arthritic (ART) mice. Paws analysed from arthritic mice had a clinical score of 0 (unaffected – UN) or 2 (affected – AFF). Statistical differences were determined by ANOVA with Newman-Keuls Multiple Comparison Test; $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

3.3. Cathepsin L expression and activity in CIA

As an interaction between CTSL and the p41 splice variant has been observed [4], a determination of the level of *Ctsl* gene expression was made in murine arthritic tissues and in Mφ generated *in vitro*. Compared with splenocytes from naïve mice, cells from spleens of arthritic mice had significantly reduced expression levels of *Ctsl* (Fig. 2A) however, this trend was not observed in lymph nodes. The expression of *Ctsl* mRNA was highest in severely affected paws compared with unaffected or naïve paws; a small but significant increase in expression was measured in unaffected paws from arthritic mice, compared to naïve paws.

Acute stimulation of murine M and GM-Mφ with TNF illustrated differences in the *Ctsl* gene expression kinetics between these cell types, with GM-Mφ expressing consistently greater levels (Fig. 2B). While GM-Mφ *Ctsl* mRNA expression continued to increase over 20 h, M-Mφ *Ctsl* mRNA expression peaked at 2 h and was declining at 20 h. In culture medium, Ctsl protein from TNF-stimulated GM-Mφ was higher than from M-Mφ. Although the presence of Ctsl protein in the medium of GM-Mφ persisted until 6 h post-stimulation (Fig. 2C), the enzymatic activity was highest at 4 h (Fig. 2D). Furthermore, the enzymatic activity was higher in GM-Mφ conditioned medium at 4 and 6 h post stimulation, compared with M-Mφ.

3.4. Increased CD74 in monocytes is a feature of the response to TNF blockade

In RA patients treated with TNF blockade, the expression of total CD74 and CD74 isoform a in monocytes was significantly lower in non-responders compared with responders after treatment (Fig. 3A and B). Responders also had an increase in total CD74 expression after treatment compared with baseline levels (Fig. 3B). The ratio of CD74 isoform a to total CD74 reflected the trend of increased expression in responders but conversely in non-responders the expression was slightly decreased after treatment (Fig. 3C). The expression of CTSL was unchanged by treatment and was not different between responders and non-responders (data not shown) however, there was a strong correlation between expression of CTSL and either total CD74 or isoform a before and after treatment when analysing all patients (Fig. 3D).

3.5. CD74 isoform a is increased in human RA synovium

To corroborate the results from experimental arthritis in human RA, the mRNA expression level of total CD74 was measured in human RA synovial tissue, with OA synovial tissue as a comparative patient group (Fig. 4A and B). There was significantly greater expression of CD74 isoform a ($p = 0.038$) in RA explants (Fig. 4A) with a trend towards higher expression in RA synovium for combined CD74 isoforms (Fig. 4B). There was a correlation in the gene expression of isoform a (relative to total CD74) in synovial cells, and the enzymatic activity after culture (Fig. 4C).

4. Discussion

Several studies have indicated that CD74 and CTSL are associated with the pathogenesis of diseases such as cancer, autoimmune diseases and atherosclerosis [20,21] but few studies have attempted to focus on

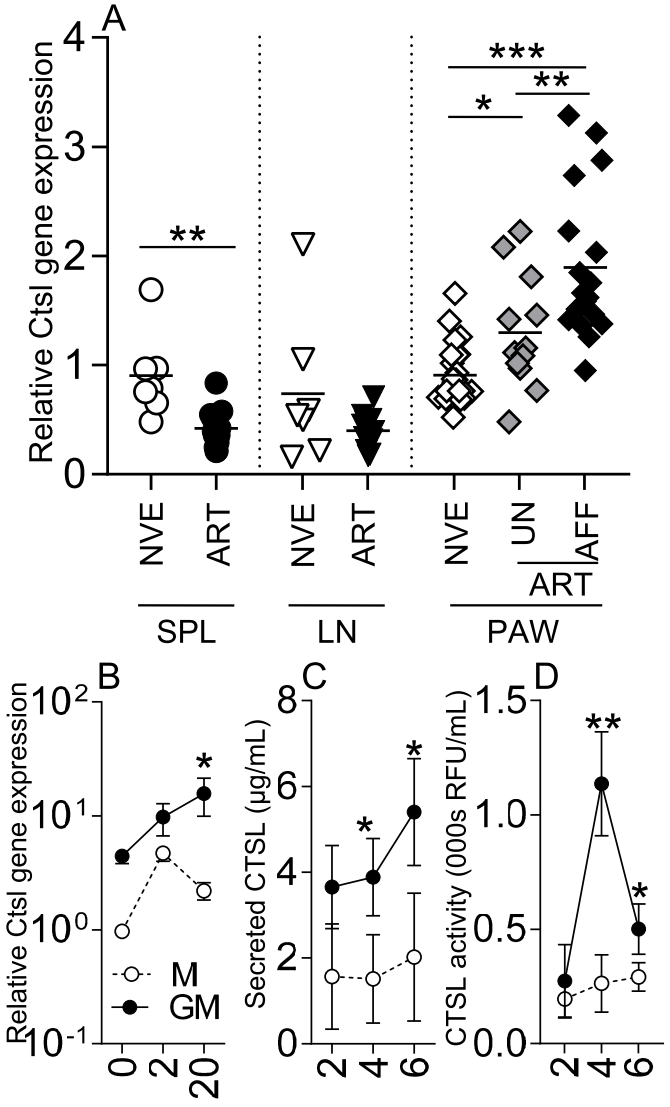

Fig. 2. Expression of *Ctsl* in murine arthritis and *in vitro*. Gene expression of (A) total CD74 and of the two different isoforms (B) CD74 p31 and (C) CD74 p41 was measured and the ratio of (D) p41:p31 gene expression was calculated in spleens (SPL), lymph nodes (LN) and paws (PAW) of naïve (NVE) and arthritic (ART) mice. Paws analysed from arthritic mice had a clinical score of 0 (unaffected – UN) or 2 (affected – AFF). Statistical differences were determined by ANOVA with Newman-Keuls Multiple Comparison Test; $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

the splice variants of CD74 and their relationship with CTSL. As the expression of CD74 is greatly increased in some forms of cancer, it has been therapeutically targeted with toxin-conjugated antibodies [22]. The pro-inflammatory activity of MIF may also potentiate or exacerbate the development of neoplasia by binding to CD74 [23]. Due to the known interaction between the p41 isoform and CTSL [3], we investigated the relationship between CD74 splice variants, CTSL and disease activity in a murine model of RA and clinical samples from patients with RA.

4.1. Experimental arthritis

Changes in the expression of CD74 splice variants were observed in

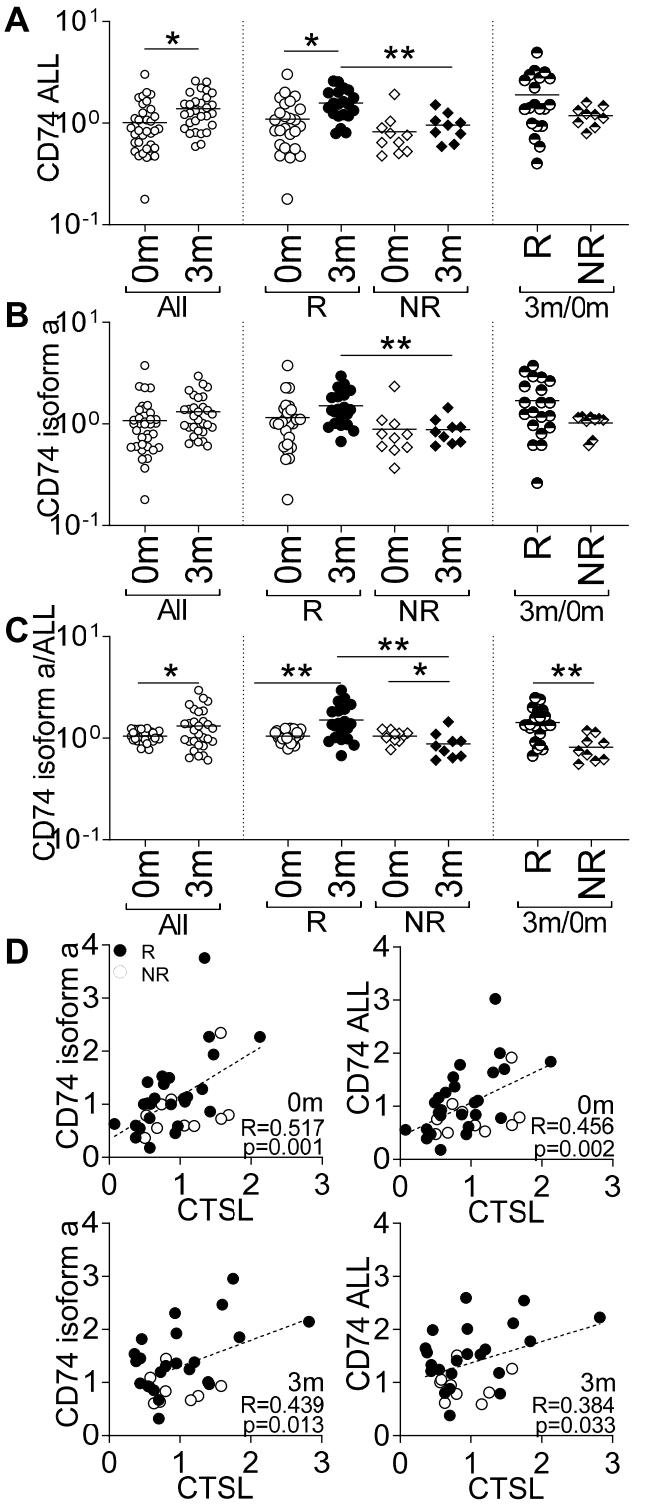

Fig. 3. Anti-TNF responder RA monocytes have increased CD74 expression. Gene expression of CD74 for (A) all isoforms and (B) CD74 isoform a. (C) Ratio of CD74 isoform a vs total CD74 isoforms gene expression. (D) Expression of *CTSL* correlated with CD74 total isoforms and CD74 isoform a (Pearson correlation). For graphs A-C, the data are as follow – all patients pre-treatment (0 months, 0 m), all patients post-treatment (3 months, 3 m), responders (R) 0 m, responders 3 m, non-responders (NR) 0 m, non-responders 3 m, change in expression (3/0 m) for responders and non-responders; $*p < 0.05$, $**p < 0.01$, *t*-test.

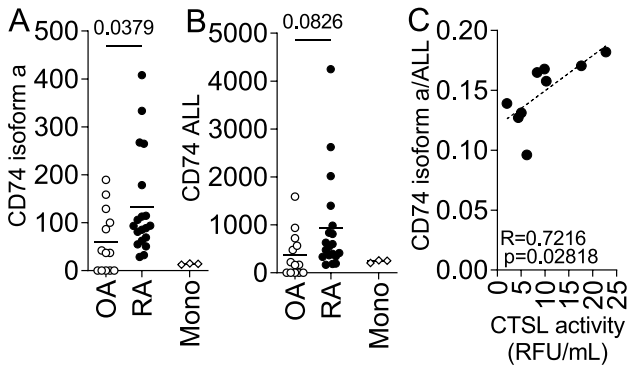

Fig. 4. Human CD74 isoform a is upregulated in RA synovial tissues. Using standard curve qPCR, the expression of (A) isoform a and (B) all CD74 isoforms was measured in human osteoarthritis (OA), rheumatoid arthritis (RA) synovial explants and unstimulated human monocytes from healthy donors (Mono). (C) The enzymatic activity of cathepsin L (CTSL) in conditioned medium from RA explants was correlated with the CD74 isoform a/all isoforms ratio; *p* values were determined using Pearson's 2-tailed correlation, n = 9 donors.

spleens, lymph nodes and paws. A positive correlation between the expression of p41 and TNF in arthritic mice was observed in paws from arthritic mice; the strongest association was found in severely affected paws. *In vitro* studies confirmed that the M1-like GM-Mφ had highest expression of CD74 and produced significantly more CTSL in response to TNF. These cells bear some resemblance to macrophages primed with IFNγ/LPS, which are canonical inducers of the M1 phenotype; IFNγ has long been known to up-regulate MHC-II genes, including CD74 [24,25], especially in combination with TNF [26].

As the spleen is a reservoir for the majority of blood monocytes in the steady state [27], changes in the expression of the p31 isoform and CTSL may reflect egress of leukocytes, particularly monocytes, from the spleen, or changes to splenic haematopoietic processes, during CIA. B cells, which have a lower p41:p31 ratio compared with myeloid APC, are also prevalent in the spleen and may contribute to the changes observed in the expression of CD74. Lymph nodes, in contrast, are a site of myeloid DC trafficking during CIA and the increase in CD74 expression, compared with naïve lymph nodes, is to some degree a function of differences in cell populations. As shown in Sup. Fig. 2, the expression of CD74 was significantly increased in bone marrow-derived DC compared with M2-like M-Mφ, and cells with the phenotype of the former would be more prevalent in draining lymph nodes during immunological activation. In order to confirm that Mφ differentiated in M-CSF were refractory to increases in CD74 expression, human MDM differentiated in M-CSF were treated with a range of stimuli. Significantly, M–CSF–differentiated Mφ from both human and mouse did not increase expression of CD74 in response to an inflammatory stimulus. Mediators associated with induction of an M2/TAM phenotype had reduced CD74 compared to unstimulated cells, with dexamethasone causing the greatest reduction in expression of the stimuli tested (Sup. Fig. 2) as has been observed for other MHC–II–associated genes [28].

The composition of Mφ phenotypes is hypothesised to change from a pro-inflammatory phenotype to a more regulatory phenotype during the course of chronic inflammation [29] and the differential expression of genes measured *in vitro* may be reflected in tissues with different disease status and cellular composition. The length of exposure to colony stimulating factors or inflammatory mediators, and degree of maturation when stimulated, may also influence Mφ phenotypes [30,31]. The higher expression levels of CD74 may also be a result of cell activation in the pro-inflammatory milieu within the lymph node or inflamed joint, e.g. the presence of IFNγ-producing cells may potentiate MHC-II -associated gene expression in the lymph nodes [32]. Despite the overlap in the range of CD74 expression between naïve and arthritic paws, in the most severely affected paws there was an increase in the p41 splice variant compared with naïve and unaffected paws. In arthritic mice, an association between the expression of the p41 splice variant and TNF was observed; the correlation was strongest in the most severely affected paws, which exhibited increased TNF expression. Experimental arthritis performed in CTSL-deficient mice has suggested that CTSL promotes disease via the thymic control of T helper cell repertoire and phenotypes [33]. While the role of CD74 and CTSL has been demonstrated in T cell selection [34,35], expression in myeloid cells at the site of inflammation is evidence of multiple roles for CTSL in the immune response [36,37].

Increased expression of MHC-II associated genes, including CD74, has been measured in monocytes treated with GM-CSF [38] and our data confirm the role of this cytokine in up-regulating the p31 and p41 splice variants in murine Mφ differentiated with GM-CSF; in M-CSF differentiated human MDM we did not observe an increase in expression after short-term GM-CSF stimulation. The consequences of increased CD74 expression in Mφ may include changes to antigen presentation and cell motility [10], and the data shown here indicate that the increased CTSL expression is not confined to lysosomal compartments but, in response to TNF, it is secreted and enzymatically active; the reduction in enzymatic activity at later timepoints may be due to the half-life of CTSL [39] or secretion of inhibitors of protease activity [40,41]. Our *in vivo* and *in vitro* results confirm the link between Mφ phenotype, p41 isoform expression and the secretion of CTSL, and demonstrate the increased expression of p41 in tissues with higher disease activity.

4.2. Rheumatoid arthritis

Interestingly, greater expression of CD74 in circulating monocytes after anti-TNF therapy was associated with a beneficial clinical outcome. Several studies have suggested differences in monocyte subset composition between responders and non-responders [42–44], with the consensus being that the $CD14^{hi}CD16^{+}$ intermediate monocyte subset is expanded in non-responders. In healthy donors, the expression of CD74 has previously been found to be increased in the intermediate monocyte subset [45]. However, in our non-responder cohort we observed slightly lower expression of CD74, compared with responders. While healthy donors with higher proportions of intermediate subset monocytes would be expected to have greater overall CD74 gene expression in the total monocyte population, an arthritic patient's monocyte subset phenotypes may be perturbed by disease activity or treatment to a greater degree than observed with normal homeostatic processes [46,47].

TNF blockade has a notable demarginating effect due to changes in the expression of selectins on endothelial cells [48]. Therefore, it is possible that circulating monocytes from RA responders have an activated, pro-inflammatory phenotype, as indicated by higher p41 expression, but their egress from the circulation is hindered by reduced endothelial selectin expression caused by the demarginating effects of TNF blockade at the later time point; in non-responders, leucocyte egress may be attenuated to a lesser degree leading to a poor response to TNF blockade and fewer monocytes with increased CD74 expression remaining in the circulation. In addition, the demarginating effects of TNF may disproportionally affect $CD16^{+}$ monocytes, which preferentially associate with the endothelial wall [49]. Alternatively, differences between responders and non-responders in cytokine expression may modulate the expression of CD74 expression; for example, higher IFNγ levels have been observed in RA anti-TNF responders [50] which may increase CD74 expression [32].

We and others have observed a paradoxical increase in inflammatory biomarkers in patients undergoing TNF blockade treatment, such as increased production of IL12/23 from ex vivo cultures (whole blood, monocytes and PBMC) [51], increased circulating Th17 [52,53] and the accumulation of Th17 in the lymph nodes during experimental arthritis [54]. In particular, the increased GM-CSF measured in plasma and the lymphocytes of responders to TNF blockade in RA [55] would potentiate

an increase in monocyte CD74 expression in responders. While the precise mechanism is not immediately apparent, changes in the CD74 splice variant ratio are a feature of the response or non-response to anti-TNF treatment and, given the association of isoform a with inflammation, indicate a further paradoxical change that occurs in patients treated with TNF blockade.

The increased expression of CD74 observed in arthritic paws was also observed in human RA synovial tissues. Previous studies have measured expression and activity of cathepsins in human RA joints and synovial fluid compared to OA joints [56,57]. Whether measured at the level of gene expression [58], protein expression [59] or enzyme activity [60], RA joint tissue tends to have higher expression of cathepsins, including CTSL, compared to OA joint tissue.

Our findings demonstrated increased expression of CD74 isoform a, which facilitates CTSL secretion, in RA synovium compared to OA synovium. CTSL enzymatic activity in the explant supernatant and the expression of CD74 isoform a were also correlated in RA samples.

5. Conclusions

We have demonstrated an association of the murine p41 splice variant with disease activity and the expression of TNF in collagen-induced arthritis. In human disease, the expression of isoform a was greater in RA synovia compared to OA synovia, and the RA synovial samples with proportionally more isoform a expression had greater CTSL enzymatic activity. The expression of isoform a tended to be higher in circulating monocytes in RA patients that responded to anti-TNF therapy. These data indicate that the CD74 p41 splice variant is strongly associated with disease activity in the joints of RA patients and paws of arthritic mice, and that differential modulation of the CD74 splice variant ratio in RA circulating monocytes is a feature of TNF blockade responder/non-responder endotypes. These results may aid the identification of underlying mechanisms of response/non-response to TNF blockade, and improve the therapeutic targeting of disease-associated processes in inflammatory auto-immune disease.

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Author statement

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Declaration of competing interest

None.

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Appendix A. Supplementary data

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