

The CN-index of hnRNP-DL Predicts an Individual “Window of Treatment Success” in RA Patients

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Abstract

Background

There is a need for biomarker to identify patients 'at risk' for rheumatoid arthritis (risk-RA) and to better predict the therapeutic response and in this study we tested the hypothesis that novel native and citrullinated heterogeneous nuclear ribonucleoprotein (hnRNP)-DL autoantibodies could be possible biomarkers.

Methods

Using Protein microarray and ELISA, epitope recognition against hnRNP-DL was analysed in sera from different developed RA disease and diagnosed SLE patients. Toll-like receptor (TLR) 7/9 and myeloid differentiation primary response gene 88 (MyD88)-dependency were studied in sera from murine disease models. HnRNP-DL expression in cultivated cells and synovial tissue was analysed by indirect immunofluorescence, immunoblot and immunohistochemistry.

Results

hnRNP-DL was highly expressed in stress granules, citrullinated in the rheumatoid joint and targeted by autoantibodies either as native or citrullinated proteins in patient subsets with different developed RA disease. Structural citrullination dependent epitopes (SCEs) of hnRNP-DL were detected in 58% of the SLE patients although 98% of these sera were α -CCP2-negative. To obtain a specific citrullinated signal value, we subtracted the native antibody value from the citrullinated signal. This CN_{DL} (Citrullinated-Native-hnRNP-DL)-index identified and the bioinformatic value was explored, as a new value for an "individual window of treatment success" in early RA and for the detection of RF-IgM/ α -CCP2 seronegative RA patients (24-46%). Negative CN_{DL} -index was found in SLE patients, risk-RA- and early RA-cohorts such as EIRA where the majority of these patients are DAS28-responders to methotrexate (MTX) treatment (87%). High positive CN_{DL} -values were associated with more severe RA, shared epitope and parenchymal changes in the lung. Specifically, native α -hnRNP-DL is TLR7/9-dependent, associated with pain and ROC-analysis revealed an association to initial MTX or etanercept treatment response, especially in seronegative RA patients.

Conclusion

CN_{DL} -index defines patients is a possible biomarker for develop RA and the "window of treatment success" thereby potentially closing the sensitivity gap.

Background

More than 20 years ago heterogeneous nuclear ribonucleoprotein (hnRNP) complexes were first described as autoimmune targets[1, 2]. These complexes associate with DNA and RNA and can stimulate Toll-like receptor (TLR) 7 and 9[3–7]. Antibodies against these structures are characteristic for autoimmune disorders, such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (scleroderma), primary Sjögren´s syndrome, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), multiple sclerosis (MS) and rheumatoid arthritis (RA) as well as for mouse models of lupus and arthritis[8–10].

In RA, the most specific antinuclear reactivity is directed against hnRNPs. Most prominent targets are hnRNP-A1 and -A2/B1 proteins, which with -A3 and -A0 proteins form the subgroup of hnRNP-A/B proteins[11–15]. Autoantibodies against hnRNP-A2/B1 (RA33) occur in about 20–40% of RA, SLE and mixed connective tissue disease (MCTD) patients[16]. Autoantibodies to hnRNP-A1 can be found in RA, SLE and MCTD, but probably are cross-reacting α -hnRNP-A2/B1 antibodies[17]. Although hnRNP-A2/B1 is citrullinated in the rheumatoid joint, and it can be targeted either as a citrullinated and or native protein in distinct subsets of RA patients[18].

Previously, we have described autoantibodies directed to the TNF α regulatory protein hnRNP-D (AUF1) to occur in 33% of SLE, 20% of RA, and 17% of MCTD patients[19]. Although predominantly localized in the nucleus, hnRNPs are exported additionally into the cytosol, where they form new autoimmune target structures in stress granules, P-bodies or RNA transport particles[19–21].

The hnRNP-D-like protein (hnRNP-DL) protein, which is also known as JKTBP, is related to the autoantigen hnRNP-D/AUF1. Due to its binding properties and structural features[22], hnRNP-DL,-D and -AB- form the D-subgroup of hnRNPs. These proteins exhibit a modular structure and conserved residues, two adjacent RNA binding domains (RBD) followed by a glycine-rich C-terminal auxiliary domain. However, they are very distinct in each of the unique N-terminal regions[23, 24].

HnRNP-DL acts as a transcription factor[25], participates in metabolism and biogenesis of mRNA[3], is able to shuttle between the nucleus and the cytoplasm and binds both to nuclear and cytoplasmic mRNAs[24], especially when containing AU-rich elements (AREs) as found within the 3'-UTR of many proto-oncogenes and cytokine mRNAs[26, 27]. Up to now, three alternatively spliced hnRNP-DL transcript variants have been described, hnRNP-DL isoform 1–3, whereas proteins only were described for isoform 1 and 2[23]. Splenocytes from pristane-primed rats restimulated with hnRNPs (-A1,-A2/B1 and -A3) induce a highly inflammatory and erosive arthritis in naïve recipient rats[6]. Furthermore, human TNF α -transgenic mice, which develop a massive erosive inflammatory polyarthritis, generate α -hnRNP-autoantibodies[28]. This supports the hypothesis of a pathogenic role of native hnRNPs in erosive arthritis and suggests that autoimmunity to nucleic acid-associated autoantigens has the potential to contribute to RA development[18]. HnRNPs may also induce pro-inflammatory cytokines, relevant for arthritis development in rats, which involve TLR7 and TLR9 but not TLR4[6].

For α -hnRNP-A2/B1, clinical associations have already been shown for RA severity, with antibodies against the citrullinated protein occurring more frequently in erosive RA and antibodies against the native protein in milder disease[18, 29]. For citrullinated peptides it has already been shown that the formation of a delta value with the corresponding arginine peptide increased diagnostic sensitivity and indicated association to shared epitope (SE) [30].

In our study, the delta value of ELISA signals was evaluated as a possible biomarker to obtain a new clinical value, as the difference between the α -citrullinated and α -native protein value. hnRNPs were further investigated in the immunopathogenesis of RA, demonstrating the clinical relevance of autoantibodies, for predicting therapeutic success, early parenchymal changes in the lung, and SE in RA. For the first time, structural epitopes resulting from the citrullination process were investigated.

Material And Methods

This paragraph is integrated in the Additional file 1.

Results

Protein macroarray screening identifies the hnRNP-DL protein as a novel autoantigen targeted in rheumatoid arthritis (RA).

Sera from 26 RA patients and 40 control subjects, including osteoarthritis (OA) patients (n = 20) and self-reported healthy blood donors (n = 20), were analysed on protein macroarrays[31]. The 20 most sensitive autoantigens only found in the RA group are listed in the Additional file 2.

We identified α -hnRNPD_L with second highest intensity score. hnRNP-A2/B1 and hnRNP-D (AUF1), have already been described as autoantibody targets in RA[11, 19]. Structure of hnRNP-DL and sequence alignment with hnRNP-D is shown in Additional file 1: supplementary Fig. 1. One of two different hnRNP-DL clones, expressing the protein fragment from amino acid 81 to 420, revealed autoantibody reactivity in 20% of RA sera (Additional file 2). This hnRNP-DL fragment was termed hnRNP-DL_{mir} (major immunogenic region). Isoform hnRNP-DL2 (amino acid 120–420) could not be detected by RA sera.

Autoantibodies against native and citrullinated hnRNP-DL are predominantly present in sera of systemic lupus erythematosus (SLE) and RA patients.

To verify the results from protein macroarray screening, hnRNP-DL_{mir} was expressed in E. coli BL21(DE3)pLysS, purified and tested for reactivity in ELISA as native (DL) and citrullinated protein version (cit-DL), using 1010 sera obtained from Risk-RA cohort (n = 71), from early RA cohorts (LURA n = 106; EIRA n = 404), from an established RA cohort (Predict n = 127), control cohorts of other autoimmune diseases (n = 216) and from healthy controls (n = 86). The majority of α -DL was found in sera of patients with SLE (34%) and RA (6–21%) and in patients with psoriasis arthritis (15%), patients with MS (5%) and scleroderma (5%) as well as healthy controls (2%) (Fig. 1B; Additional file 1: supplementary Fig. 2). Interestingly, we obtained very different sensitivities within the four investigated RA cohorts whereby Risk-RA- (13%) and EIRA cohort (21%) showed the highest sensitivities. Cohorts under certain therapy or advanced disease duration showed lower values (LURA 8%/Predict 6%).

Since citrullinated antigens, among them hnRNP-A2/B1[18], are the most specific targets in RA, we analysed autoantibody responses against cit-DL, with the highest signaling and positivity found in the early and established RA cohorts (64–100%). With special focus on the seropositive and seronegative RA patients only α -cit-DL signals differ significantly within all investigated RA cohorts, not α -DL values (Fig. 1A/B; Additional file 1: supplementary Table 1). Although α -cit-DL signals of seronegative patients were lower than those of seropositive patients, they were still significantly higher than in other diseases in EIRA and Predict cohort (Additional file 1: supplementary Table 2).

Noticeable 58% of the SLE patients in comparison to other diseases were α -cit-DL positive (α -DL 18%), although 98% of the tested SLE sera were α -CCP2-negative. We determined the difference between the ELISA signals, to get a value that describes the relationship between α -cit-DL and α -DL. This value we named CN_{DL}-Index (Δ DL), shown in Fig. 1C, with the highest values detected in the RA cohorts. In established RA (Predict) the highest CN_{DL}-Index (sensitivity: 100%/72% vs. healthy controls/other diseases) and exclusively positive values were detected. In contrast, 11–20% of early RA patients (Risk-RA/EIRA/ LURA) had a negative CN_{DL}-index, where α -DL was higher than α -cit-DL. Besides, only SLE patients and single exceptions in other diseases had a negative CN_{DL}-index below

- 0.1. In the early RA cohort EIRA the CN_{DL}-Index correlated positively to α-cit-DL and there, exclusively in the seronegative EIRA negatively to α-DL response (Additional file 1: supplementary Fig. 3).

Figure 1. Distribution of ELISA signals of α-hnRNP-DL_{mir} autoantibodies. reactivities were predominantly found in SLE and RA.

A-C, Prevalence of citrullinated α-hnRNP-DL_{mir} (cit-DL) (**A**), α-hnRNP-DL_{mir} (DL) (**B**) and the difference between cit-DL and DL signal (ΔDL) (**C**) in sera from Risk-RA patients (n = 71), early RA patients (LURA n = 106; EIRA n = 404), established RA patients (Predict n = 127), SLE patients (n = 89), other diseases (n = 127) and healthy controls (n = 86) determined by ELISA. The dotted lines mark the cutoff versus other diseases (except SLE) or healthy controls with 98% specificity each. OD, optical density; SLE, systemic lupus erythematosus

Anti-cit-DL and CN_{DL}-Index correlated with parenchymal changes in lung/shared epitope and identified people at risk to develop RA.

Anti-DL autoantibodies were detectable in early RA. Therefore, we investigated aCCP2-positive healthy subjects with musculoskeletal symptoms, classified as Risk-RA cohort, differentiating between subjects developing arthritis during follow-up and those remaining healthy without arthritis diagnosis. Further we analysed α-DL autoantibody association with certain risk factors for RA. We plotted respectively α-cit-DL, α-DL and the CN_{DL}-index in the LURA cohort with the parenchymal changes in the lung and in the EIRA cohort with the genetic risk factor shared epitope.

In the Risk-RA cohort α-cit-DL and CN_{DL}-Index were significantly elevated in progressors (Fig. 2A), in the LURA cohort in patients with parenchymal lung changes (Fig. 2B) and in the EIRA in patients with shared epitope, particularly in those carrying two copies (Fig. 2C). No significant differences were found for α-DL antibodies.

Figure 2. Anti-citrullinated hnRNP-DL_{mir} autoantibodies are detectable even before the onset and in early status of disease. **A-C,** Anti-citrullinated hnRNP-DL_{mir} (cit-DL), α-hnRNP-DL_{mir} (DL) and Δ OD between cit-DL and DL (ΔDL) were measured by ELISA. **A,** In Risk-patients of arthritis the OD-levels of citDL and ΔDL before onset are significantly specific in the patient group where the arthritis has already been diagnosed compared to the group without diagnosis (n = 71; non Arthritis n = 34/Arthritis n = 37; MannWhitneyU; citDL median_{non Arthritis}=0.19/median_{Arthritis}=0.46; p = 0.0006; NC-index median_{non Arthritis}=0.10/median_{Arthritis}=0.38; p = 0.0003). **B-C,** Cit-DL and ΔDL are significantly associated with parenchymal changes in the lung of early RA patients of the LURA cohort (**B;** n = 106; no n = 48/PC n = 58; MannWhitneyU; citDL median_{no}=0.23/median_{PC}=0.53; p = 0.0340; NC-index median_{no}=0.16/median_{PC}=0.44; p = 0.0332) and with and shared epitopes of the early RA patients of the EIRA cohort (**C;** n = 404; no n = 112/SE n = 213/double SE n = 79; MannWhitneyU; citDL median_{no}=0,27/median_{SE}=0.36; p = 0.0003, median_{no}=0,27/median_{double SE}=0.54; p < 0.0001, median_{SE}=0,36/median_{double SE}=0,54; p = 0.0453; NC-index median_{no}=0,11/median_{SE}=0.21; p < 0.0001, median_{no}=0,11/median_{double SE}=0.34; p < 0.0001, median_{SE}=0,21/median_{double SE}=0,34; p = 0.0061).

Mann-Whitney U test was performed for analysing significance of indicated groups (*p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001). OD, optical density; nm, nano meter; ns, not significant; PC, parenchymal changes in lung;

High α -DL autoantibody levels found in 6-month EULAR responders for MTX or Enbrel® treatment

We examined our biomarkers (α -cit-DL, α -DL and CN_{DL} -index) with therapy data of the EIRA and Predict cohort. 192 MTX treated EIRA patients were analysed (Fig. 3A-C; Additional file 1: supplementary Fig. 4A-C). The ROC analysis of α -DL signals reached 12% sensitivity with 90% specificity, using the RA-specific cutoff level (OD 0,371) for detecting MTX response. ROC results got more significance for detecting MTX responses in the seronegative group (cutoff 0,371; 16% sensitivity, 94% specificity; Additional file 1: supplementary Table 6).

Because α -DL correlated negatively to the CN_{DL} -Index in the seronegative group (Additional file 1: supplementary Fig. 3), we analysed MTX-treated EIRA patients with negative CN_{DL} -index. 87% of these patients were responders. We reached sensitivities in a range of 15–33% (100/75% specificity) to detect MTX response (Additional file 1: supplementary Table 7).

In Predict cohort (Enbrel®-treatment) no CN_{DL} -index/response association were found since all patients had equally high positive CN_{DL} -index and none of them negative values. ROC analysis of α -cit-DL or CN_{DL} -index showed no specific response cutoff. But with α -DL we identified 23% of the EIRA patients as MTX responder and in the seronegative group 25% (90% specificity). Among the established RA cohort (Predict) α -DL reached 13% sensitivity and even 25% within the seronegative group for the detection of Enbrel® response (100% specificity; Fig. 3D-F; Additional file 1: supplementary Table 8).

Figure 3. Diagnostic performance of α -hnRNP-DL_{mir} (DL) for the detection of therapy response.

High baseline titer against α -hnRNP-DL_{mir} (DL) is rather present in 6-month EULAR Responder RA patients who had received MTX or α -TNF inhibitor therapy (Enbrel®). **A-C**, α -DL were measured by ELISA in patient sera from the EIRA cohort treated with MTX (n = 192) with 161 EULAR Responder and 31 EULAR non-Responder among 6 months. Above these values ROC analyses were performed for detecting DAS28 therapy response. **D-F**, α -DL were measured by ELISA in patient sera from the Predict cohort treated with Enbrel® therapy with 6-month EULAR response data (n = 94, responder n = 63, non-Responder n = 31). Based on the signals, ROC analysis was performed for detecting DAS28 therapy response.

OD, optical density; nm, nano meter; vs., versus; RA, rheumatoid arthritis; MTX, Methotrexat; seropos., rheumatoid factor IgM and/or α -CCP2 positive patients; seroneg., rheumatoid factor IgM and α -CCP2 negative patients.

Anti-cit-DL and α -DL increase the serodiagnostic sensitivity in early RA.

All RA cohorts were analysed to determine diagnostic sensitivities of α -cit-DL and α -DL, in RF IgM/ α -CCP2-seropositive and -negative patients.

The calculated cutoff versus healthy controls (96% specificity), identified 80% of the subjects in the Risk-RA cohort, which are exclusively α -CCP2-positive. In the LURA/ EIRA cohort 32/73% of the seronegative patients were identified. In the Predict cohort all patients could be identified with one of our biomarkers and α -DL response was

on average the lowest (6%). In SLE 84% in total were detected (α -DL: 34%; α -cit-DL: 80 %). In other autoimmune diseases about half (48%) of the patients were detected in total with our biomarker set.

Using the cutoff versus other diseases (96% specificity), we detected 51% of the seronegative established RA patients and 8–17% of the early RA patients (Table 1).

Table 1

Sensitivity of α -(cit)hnRNP-DL_{mir} (α -DL) autoantibodies in sera from Risk-RA patients, early RA patients (LURA/EIRA), established RA patients (Predict), SLE patients (n = 89), other diseases and healthy controls determined by ELISA. The sensitivity is indicated in percent with 98% specificity versus healthy controls (left of slash) and versus other diseases SLE (right of slash). Total DL is the combined reactivity and describes the percentage of patients reacting positively to at least one of the three biomarkers α -cit-DL, α -DL and Δ DL.

	RF and/or CCP positive				RF and CCP negative							
	cit-DL	DL	Δ DL	total DL	cit-DL	DL	Δ DL	total DL	cit-DL	DL	Δ DL	total DL
RiskRA	n = 71				n = 71				n = 0			
% pos.	70/28	13/6	68/34	80/39	70/28	13/6	68/34	80/39	-	-	-	-
LURA	n = 106				n = 81				n = 25			
% pos.	64/38	8/4	64/40	69/42	77/48	7/2	77/52	80/53	24/4	12/8	24/0	32/8
EIRA	n = 404				n = 202				n = 202			
% pos.	80/33	21/9	68/31	84/40	94/59	17/5	89/59	96/63	65/7	25/14	46/2	73/17
Predict	n = 127				n = 86				n = 41			
% pos.	100/67	6/0	100/72	100/72	100/77	6/0	100/83	100/83	100/46	5/0	100/51	100/51
SLE	n = 89											
% pos.	80/58	34/18	72/57	84/73								
other	n = 127											
% pos.	45/2	4/2	41/2	48/4								
HC	n = 86											
% pos.	2/0	2/0	2/0	5/0								
RA: Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus												

Localization and expression of hnRNP-DL in different cell lines and synovial tissue.

Affinity-purified α -DL autoantibodies from RA patient sera were used for localization of hnRNP-DL in HeLa- and HEP-2 cells. Sparring the nucleoli in interphase cells, staining with the α -DL autoantibodies showed a

nucleoplasmic staining with large speckles (Fig. 4A-a-b). However, the nucleoplasmic staining produced by α -hnRNP-D and α -hnRNP-A2/B1 antibodies was more homogeneous (Fig. 4A-e-f) and stained as well as α -DL autoantibodies discrete cytoplasmic foci when cells were stressed by arsenite (Fig. 4A-c, -e-f). Notably, the co-localization experiment showed α -DL antibodies stained a subset of cytoplasmic stress granules (Fig. 4A-c), independent of size and localization. hnRNP-D could be detected in nearly all granules (Fig. 4A-g, yellow), like the controls Ataxin2 and RCK/p54 (Fig. 4A-d/h).

Since previous studies demonstrated hnRNP-A2/B1 and hnRNP-D to be highly expressed in synovial tissue of RA patients and arthritic mice [19, 28, 32, 33], we analysed the expression of hnRNP-DL in the human joint. Specific rabbit antibodies recognizing hnRNP-DL 1 and - 2 expression were tested by immunohistochemistry in synovial tissue of RA and OA patients and from healthy controls (Fig. 4B). These analyses revealed hnRNP-DL to be highly expressed in RA tissue. Nuclear and cytoplasmic expression was seen in cells of RA synovial tissue, in contrast to the exclusive nuclear staining observed in OA and normal tissue (Fig. 4B, arrows).

We further investigated the expression of hnRNP-DL under inflammatory conditions in IL1 α - and TNF α -stimulated HepG2-, as well as in IL6-stimulated HeLa cells by immunoblotting (Fig. 4C). TNF α and particularly IL1 α upregulate, whereas IL6 downregulates the expression of hnRNP-DL and furthermore induces its degradation.

We further detected citrullinated proteins of the same molecular weight as of hnRNP-DL (Fig. 4D) in the synovial tissue.

Figure 4. Localisation and Expression of cytokine-regulated, stress granule protein hnRNP-DL_{mir} in cells and synovial tissue. Anti-human hnRNP-DL_{mir} antibodies detect stress granules in immunofluorescence microscopy. Staining with an affinity-purified α -human hnRNP-DL_{mir} antibody was performed in HEp-2 (a) and HeLa cells (b). HeLa cells were treated with 0.5 mM sodium arsenite to induce stress granules and stained with affinity-purified α -human hnRNP-DL_{mir} antibodies (c), mouse α -human ATXN2 antibodies 63 (d), mouse α -human hnRNP-A2/B1 antibodies (e) and α -human AUF1 peptide-specific rabbit serum 19 (f). Co-localization of AUF1 and stress granules/P-bodies. Staining of HEp-2 cells with α -RCK/p54 64 antibodies (g) and double staining of HEp-2 cells with affinity-purified α -human AUF1 (green) and α -RCK/p54 64 antibodies (red) (h). Merged sections are visible in yellow. B, Expression of JKTBP in synovial tissue from a patient with rheumatoid arthritis, a patient with osteoarthritis and a healthy subject, each in 20-fold and detail in 40-fold magnification. C, Influence of cytokines on hnRNP-DL expression determined by immunoblotting. Cellular extracts from unstimulated, IL1 α - or TNF α -stimulated HeLa cells and from unstimulated and IL6-stimulated HepG2 cells were probed with α -hnRNP-DL1/2-peptide specific rabbit serum. D, Citrullination of hnRNP-DL1/2 in synovial tissue from a patient with rheumatoid arthritis was investigated with an α -deiminated arginine antibody and an α -hnRNP-DL antibody.

Anti-DL in animal models of RA and SLE with association to TLR7/9 and MyD88 - supports reference to clinical pain

Anti-citDL/ α -DL autoantibodies, in baseline samples, are associated with pain VAS after 6 months of various treatments of EIRA patients; Additional file 1: supplementary Table 3–5).

Therefore, we wanted to study the production of α -DL autoantibodies in the context of TLR and MyD88-knock-out mice, known to be involved in pain pathway [34, 35]. Because hnRNP-DL is highly conserved in human and mouse

(similarity 98,5%[36]; Additional file 1: supplementary Fig. 1), we analysed α -DL in sera of mouse models of RA and SLE (Table 2).

In zymosan-treated SKG-mice[37], α -DL autoantibodies were twice as frequent (50%) compared to the less severe arthritis model without zymosan induction (25%).

Interestingly, in the interleukin-1 receptor antagonist-deficient (IL-1Ra^{-/-})-mouse arthritis model we found high signals of α -DL autoantibodies in all mice tested.

MRL/lpr-mice produce antibodies against hnRNPs[38] and snRNPs[39] α -DL autoantibodies were detectable in 85%, while none of them were positive for the citrullinated protein version.

We analysed sera from TLR7-, TLR9- and TLR7/TLR9-double deficient lupus-prone MRL/lpr-mice. This investigation revealed that α -DL autoantibodies were TLR7/-9 dependent and only completely absent in the double deficient mice, while they remained detectable in about 50% of the single TLR7- or TLR9-knockout MRL/lpr-mice. MyD88 plays a central role in TLR-pathway[40]. We tested MyD88-deficient mice, which did not produce α -DL autoantibodies except two mice with very low titer. Further we tested knock-out mice of Toll interleukin-1 receptor 8 (TIR8, SIGIRR, IL1R8), a negative regulator of TLR-IL1-receptor family signaling. Genetic inactivation of this protein, which is associated with severe autoimmunity and high autoantibody production[41], increased prevalence of α -DL autoantibodies by 50%, with a three times higher mean level of ELISA signal intensity (Table 2).

Table 2

Frequency of autoantibodies against recombinant hnRNP-DL_{mir} in sera from different RA and SLE mouse models.

Mouse model	Model of	Autoantigen(s) assayed	No. of sera tested	% positive	‡Ratio Mean OD positive
SKG (-/+ Zymosan)	RA	hnRNP-DL _{mir}	8/8	25/50	2,48/1,23
Balb/c (IL-1Ra ^{-/-})	RA	hnRNP-DL _{mir}	36	100	7,89
MRL-lpr	SLE	hnRNP-DL _{mir} [†]	20	85	4,22
MRL-lpr (MyD88 ^{-/-})	SLE	hnRNP-DL _{mir} [†]	20	10	1,3
MRL-lpr (TLR9 ^{-/-})	SLE	hnRNP-DL _{mir}	4	50	2,42
MRL-lpr (TLR7 ^{-/-})	SLE	hnRNP-DL _{mir}	7	43	2,55
MRL-lpr (TLR7/9 ^{-/-})	SLE	hnRNP-DL _{mir}	7	0	-
C57BL/6 lpr	SLE	hnRNP-DL _{mir} [†]	12	33	2,46
C57BL/6 lpr (SIGIRR/TIR8 ^{-/-})	SLE	hnRNP-DL _{mir} [†]	12	83	6,83
C57BL/6 (-/+ R848)	TLR7/8 agonist	hnRNP-DL _{mir}	10/10	0/10	-/1,39

RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematosus; SIGIRR/ TIR8: Single Ig IL-1-related receptor/ Toll/interleukin-1 receptor 8; R848: SIGIRR TLR7/8 agonist; MyD88: myeloid differentiation primary response gene 88; TLR: Toll-like receptor. † Additionally citrullinated hnRNP-DL_{mir} were tested. In no case citrullination of hnRNP-DL_{mir} resulted in a higher signal compared to the native hnRNP-DL_{mir} form (no additional reactivity). ‡ Ratio Mean OD positive reflects the level of the positive signals in each mouse model and was calculated as the quotient of the mean value of the positive signals and the diagnostic cutoff.

Discussion

RA antibody systems are remarkably diverse, characterized by the presence of those against native proteins as well as those containing posttranslational modifications (PTMs)[42, 43]. While current models of RA have embraced PTMs as core principles of pathogenesis[42, 44], α -native protein antibodies are not adequately explained by the PTM-centric paradigm of autoantigen selection. The direct α -citrullinated protein-antibody response may depend on the presence of permissive factors, i.e. a genetic predisposition, as has been shown for α -cit-DL with its shared epitope (SE)-dependency and the continued production of modified antigen. Chronic bacterial infection, such caused by *Aggregatibacter actinomycetemcomitans*[45] or *Porphyromonas gingivalis*, which can citrullinate hnRNPs[46] or smoking[47, 48], leading to overexpression of hnRNPs, as shown by our results with overexpression and citrullination of hnRNP-DL in RA joint.

In early RA, a serodiagnostic gap of 50–60%[49–52] left by using RF IgM/ α -CCP2 assays. This is of particular importance as patients considered to be autoantibody negative may erroneously not be diagnosed as having RA

due to inappropriate therapeutic measures. In recent years, novel biomarkers have been described with sensitivities between 16–67% in α -CCP-negative RA cohorts[53]. However, the clinical utility of these biomarkers is questionable because diagnostic specificities are largely unknown and will have to be shown in further studies. RF IgM/ α -CCP2-seronegative RA patients became seropositive by a combination of our biomarker set (α -cit-DL, α -DL, CN_{DL}-index). In the clinical autoantibody testing, the new biomarker can be used for detecting people 'at risk' for RA, and for early and established RA, reducing the sensitivity gap of RF IgM/ α -CCP2-seronegative patients (sensitivity RF IgM/CCP2 negative LURA/EIRA/Predict 32%/73%/100%; Table 1).

It has already been published that it is important to study the citrullinated signal adjusted from the unmodified protein/peptide signal, to obtain the specific signal, which is added or reduced by the modification. It has been shown that these autoantibodies occur specifically in RA, but without clinical associations such as therapy response[30, 46].

Therefore, we have introduced and tested a new biomarker CN_{DL}-index which measures the difference of α -cit-DL and α -DL ELISA levels, covering both antibodies against citrullinated epitopes and structural citrullinated epitopes (SCEs). Negative CN_{DL}-index was detectable at an early timepoint of arthritis and even before arthritis starts. Moreover, RA patients with such negative CN_{DL}-index tended to respond positively to MTX/Enbrel® therapy. As RA progresses, the CN_{DL}-index became increasingly positive and was associated with SE, parenchymal changes in lung and lower the response to MTX therapy.

Citrullination is a hydrolytic reaction, the target protein mobility in SDS-PAGE will shift, yielding a noncharged citrulline amino acid and neutral urea through the hydrolysis of the strongly basic positively charged side chain of arginine by water. This charge shift affects protein structure, protein-protein interactions, and hydrogen bond formation, and it may cause protein denaturation[54, 55] This study suggests an alternative model to the PTM centric model in which the antigen is initially targeted independent of citrullination, but may be depend on a structural change induced by cryptic PTM that causes the autoantibody binding. Demonstrated by 58% detection of the SLE patients were α -cit-DL positive (α -DL 18%) versus other diseases, although 98% of the tested SLE sera tested α -CCP2-negative. Citrullination leads to formation of a new SCE, whose recognition is independent of directly targeting the citrulline site. This new form of α -SCE-autoantibodies may explain the shift from an initial native autoantibody response against PTMs. DNA, RNA and TLR7/9 activation are required to generate α -hnRNP-specific B cells and this complex induced RA in a pristane-induced arthritis model of RA[56]. Interestingly MyD88 deficiency leads to reduction of pain[34], which may explain the correlation of α -DL with pain-VAS after six months in the EIRA cohort. Autoantibodies against DL did not correlate to RF IgM or α -CCP2 or SE. These antibodies can be used specifically in the seronegative group to predict the therapeutic outcome and pain level after six months of treatment.

The α -DL autoantibody level disappeared in the course of RA, inversely the α -cit-DL autoantibody level increased, independently from the therapeutic regime. Therefore, future therapies should use tolerance induction with the native RA autoantigens in 'high-risk' individuals to inhibit the epitope spreading to PTM autoantigens, as we have found the α -DL autoantibodies in major models of SLE/RA.

Native antigens as part of stress granules are used in existing models of experimental arthritis to induce arthritis, but not the citrullinated antigens[56] SE and specific exogenous factors are missing in the studied animal models of RA and SLE, explaining the lack of ACPAs and SCE autoantibodies. Anti-native protein antibodies may

represent markers for the detection of risk people in the earliest pre disease of RA, preceding the development of the ACPA response, predicting a mild disease. For α -hnRNP-A2/B1 autoantibodies has already been published that it is also associated with less erosive disease, exclusively in early RA[18, 29]. Recently, several more reactivities against native proteins in RA have been published[43].

Therefore, it is important to measure other hnRNP-autoantibodies and in combination in future studies to evaluate them for personalized medicine.

Conclusions

We suggest that hnRNP-autoantibodies produced by patients with systemic autoimmune diseases target mRNA decay complexes which are part of stress granules. We hypothesize that increased formation and structural modification via bacterial or human enzymes of such protein complexes (e.g., in inflammatory processes with overexpression of IL1 α and/or TNF α). This may lead to pathogenic autoimmune reaction against a structurally changed native hnRNP-DL introducing the SCE epitopes before PTM specific targets increase of time. To summarize, introducing CN-index biomarker measuring the specific anti-citrulline signal in autoantigens will help to facilitate objectively early RA treatment decisions, not measurable by commercial ACPA assays.

Abbreviations

hnRNP-DL: heterogeneous nuclear ribonucleoprotein D-like (JKTBP); cit-DL: citrullinated hnRNP-DL; CN_{DL}-Index: Difference of ELISA signal of α -citrullinated and α -native-hnRNP-DL (Δ hnRNP-DL); mir: major immunogenic region; SCE: Structural citrullination dependent epitopes; PTMs: posttranslational modifications; AUF1: TNF α regulatory protein hnRNP-D; RA33: Autoantibodies against hnRNP-A2/B1; RBD: RNA binding domains; AREs: AU-rich elements; Rheumatoid Arthritis; SLE: systemic lupus erythematosus; MS: multiple sclerosis; reA: reactive arthritis; Sclero: scleroderma; Sjö: Sjögren's syndrome; PsA: psoriasis arthritis; MB: ankylosing spondylitis; OA: Osteoarthritis; HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis; MCTD: mixed connective tissue disease; SE: shared epitope; PC: parenchymal changes in the lung; MTX: methotrexate;

IL: Interleukin; TNF: tumor necrosis factor; TLR: Toll-like receptor; MyD88: myeloid differentiation primary response gene 88; SIGIRR/ TIR8: Single Ig IL-1-related receptor/ Toll/interleukin-1 receptor 8; R848: SIGIRR TLR7/8 agonist; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide electrophoresis; HC: healthy controls; ROC: receiver operating characteristic; RCK/p54: DEAD box/RNA helicase protein, rck/p54; ATXN: Ataxin-2; IL-1Ra: interleukin-1 receptor antagonist;

Declarations

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Authors' contributions

BM and KS conceived the study and design.

BM, TH, UN, SK, APC, GS, KS for acquisition of data.

DMR, MIK, KJA, AIC, VJ, APC, MJS, GS collected database and biological material.

BM, ZK, MJ, FW, GS, KS, JR involved in analysis and interpretation of data.

All authors BM, MJ, ZK, TH, FW, UN, SK, DMR, MIK, HJA, GRB, AIC, VJ, APC, MJS, JR, GS, KS were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

The study has been performed in compliance with the Declaration of Helsinki, with informed consent obtained from all study participants, and ethical approval granted at the Regional Ethical Review Board in Berlin, Erlangen, Vienna, and Stockholm.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflicting financial interest and have not received funding from commercial sources in the context of this study. KS and ZK are a co-inventors of the patent WO/2010/072673 Diagnostic Prediction of Rheumatoid Arthritis and Systemic Lupus Erythematosus, protecting the use of the hnRNPD and cit-hnRNPD in RA and SLE.

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AIC MD, PhD, professor, group leader at Karolinska Institutet died in March 2021.

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Figures

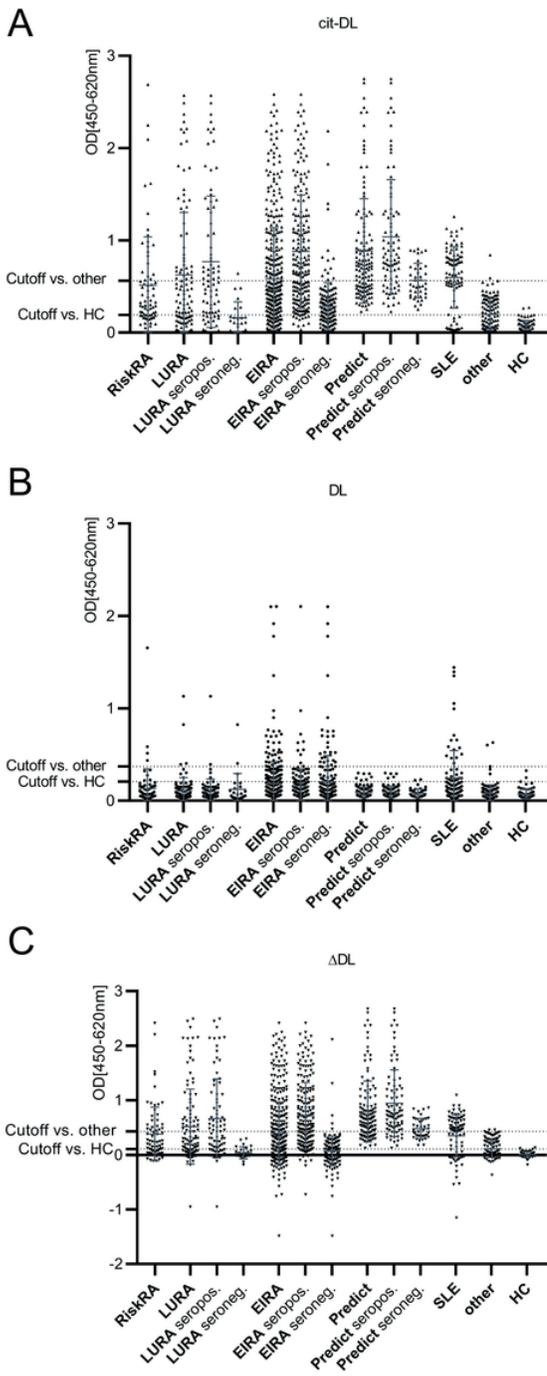


Figure 1

Distribution of ELISA signals of α -hnRNP-DLmir autoantibodies. reactivities were predominantly found in SLE and RA.

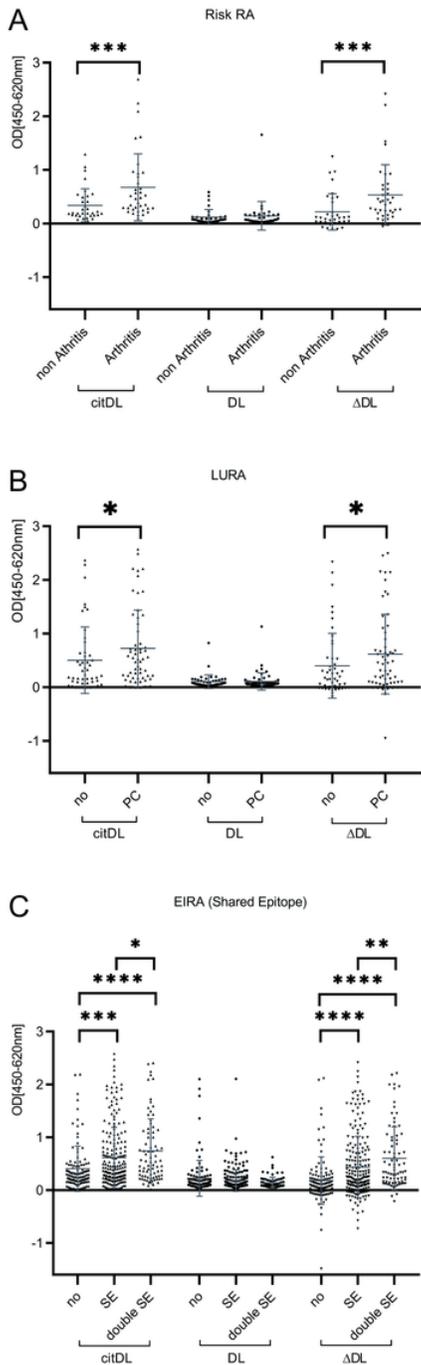


Figure 2

Anti-citrullinated hnRNP-DLmir autoantibodies are detectable even before the onset and in early status of disease. A-C, Anti-citrullinated hnRNP-DLmir (cit-DL), α -hnRNP-DLmir (DL) and Δ OD between cit-DL and DL (Δ DL) were measured by ELISA. A, In Risk-patients of arthritis the OD-levels of citDL and Δ DL before onset are significantly specific in the patient group where the arthritis has already been diagnosed compared to the group without diagnosis (n=71; non Arthritis n=34/Arthritis n=37; MannWhitneyU; citDL mediannon Arthritis=0.19/medianArthritis=0.46; p=0.0006; NC-index mediannon Arthritis=0.10/medianArthritis=0.38; p=0.0003). B-C, Cit-DL and Δ DL are significantly associated with parenchymal changes in the lung of early RA patients of the LURA cohort (B; n=106; no n=48/PC n=58; MannWhitneyU; citDL medianno=0.23/medianPC=0.53;

p=0.0340; NC-index medianno=0.16/medianPC=0.44; p=0.0332) and with and shared epitopes of the early RA patients of the EIRA cohort (C; n=404; no n=112/SE n=213/double SE n=79; MannWhitneyU; citDL medianno=0,27/medianSE=0.36; p=0.0003, medianno=0,27/mediandouble SE=0.54; p<0.0001, medianSE=0,36/mediandouble SE=0,54; p=0.0453; NC-index medianno=0,11/medianSE=0.21; p<0.0001, medianno=0,11/mediandouble SE=0.34; p<0.0001, medianSE=0,21/mediandouble SE=0,34; p=0.0061). Mann-Whitney U test was performed for analysing significance of indicated groups (*p<0.05, **p<0.01, ***p<0.001 ****p<0.0001). OD, optical density; nm, nano meter; ns, not significant; PC, parenchymal changes in lung; SE, shared epitope

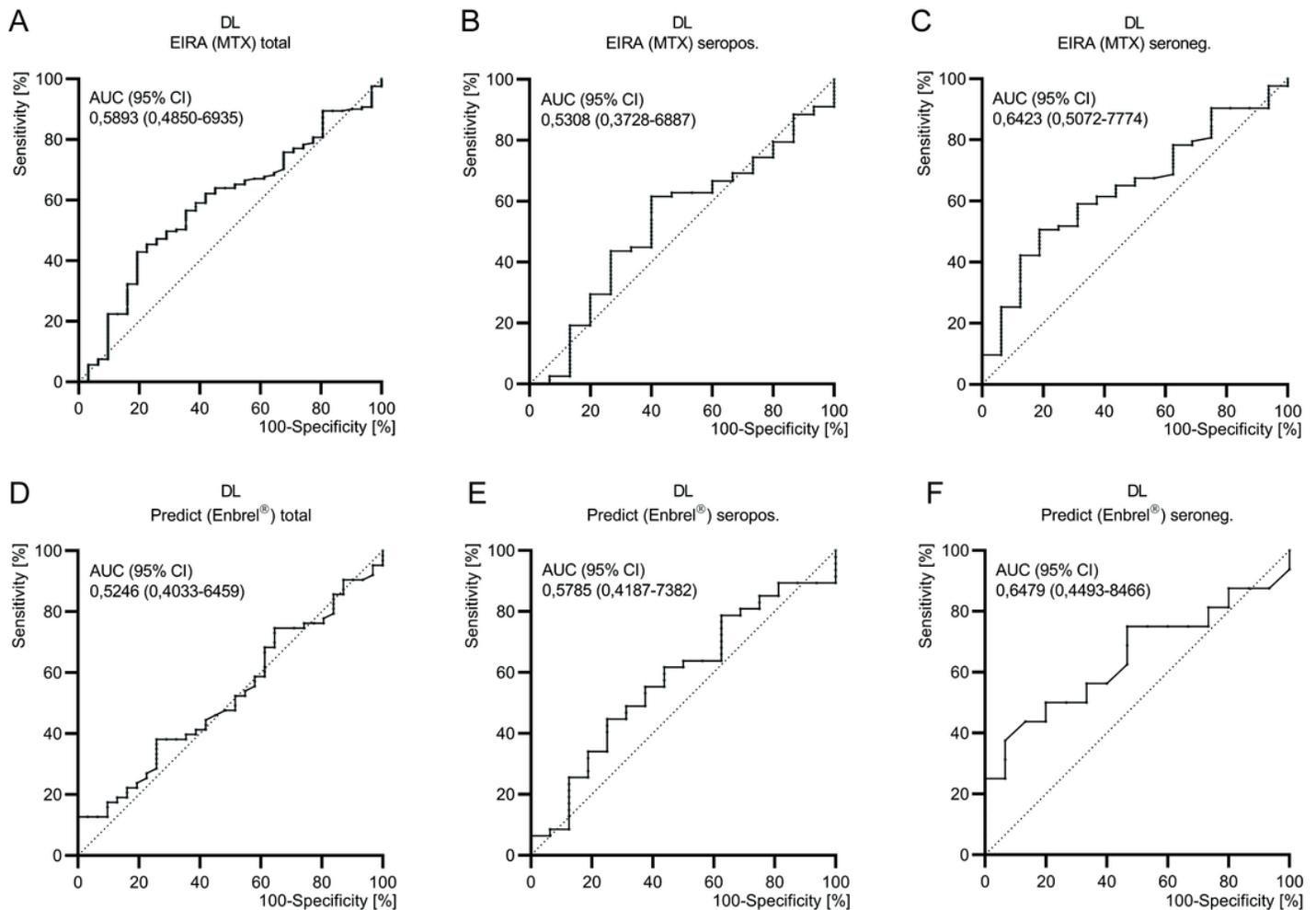


Figure 3

Diagnostic performance of α -hnRNP-DLmir (DL) for the detection of therapy response.

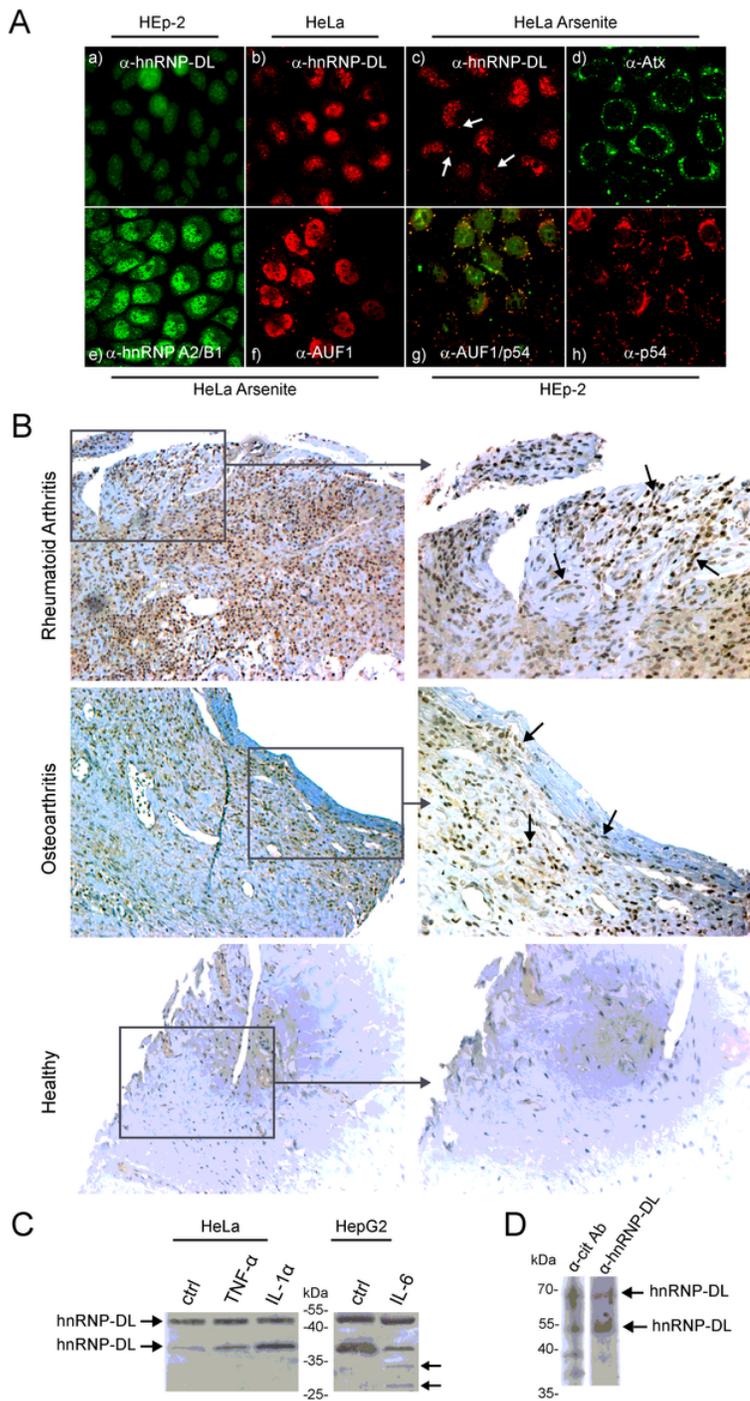


Figure 4

Localisation and Expression of cytokine-regulated, stress granule protein hnRNP-DLmir in cells and synovial tissue. Anti-human hnRNP-DLmir antibodies detect stress granules in immunofluorescence microscopy. Staining with an affinity-purified α -human hnRNP-DLmir antibody was performed in HEp-2 (a) and HeLa cells (b). HeLa cells were treated with 0.5 mM sodium arsenite to induce stress granules and stained with affinity-purified α -human hnRNP-DLmir antibodies (c), mouse α -human ATXN2 antibodies 63 (d), mouse α -human hnRNP-A2/B1 antibodies (e) and α -human AUF1 peptide-specific rabbit serum 19 (f). Co-localization of AUF1 and stress granules/P-bodies. Staining of HEp-2 cells with α -RCK/p54 64 antibodies (g) and double staining of HEp-2 cells with affinity-purified α -human AUF1 (green) and α -RCK/p54 64 antibodies (red) (h). Merged sections are visible in

yellow. B, Expression of JKTBP in synovial tissue from a patient with rheumatoid arthritis, a patient with osteoarthritis and a healthy subject, each in 20-fold and detail in 40-fold magnification. C, Influence of cytokines on hnRNP-DL expression determined by immunoblotting. Cellular extracts from unstimulated, IL1 α - or TNF α -stimulated HeLa cells and from unstimulated and IL6-stimulated HepG2 cells were probed with α -hnRNP-DL1/2-peptide specific rabbit serum. D, Citrullination of hnRNP-DL1/2 in synovial tissue from a patient with rheumatoid arthritis was investigated with an α -deiminated arginine antibody and an α -hnRNP-DL antibody.

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