# NKG2D ligands in inflammatory joint diseases: analysis in human samples and mouse models

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## Abstract

### Objective

*NKG2D ligands (NKG2DLs) are stress-inducible molecules involved in multiple inflammatory settings. In this work, we quantified MICA, an NKG2DL, in the synovial fluid of patients suffering various arthritides and measured Nkg2dLs gene expression in murine models of acute joint inflammation.*

### Methods

*Soluble MICA (sMICA) was quantified by ELISA is synovial fluids harvested from patients suffering osteoarthritis, rheumatoid arthritis, psoriatic arthritis, calcium pyrophosphate crystal arthritis, urate crystal arthritis and reactive arthritis. Transcripts encoding murine NKG2DLs were quantified by RT-qPCR in the joints of mouse models of rheumatoid arthritis, urate crystal arthritis and osteoarthritis.*

### Results

*Marked overproduction of sMICA was observed in the synovial fluid of RA patients. Mouse studies highlighted the complex transcriptional regulation of Nkg2d ligands encoding genes depending on the inflammatory setting and microenvironment*

### Conclusion

*sMICA quantification could be an interesting biomarker to identify acute inflammation in RA patients in whom classical markers (i.e. anti-citrullinated protein antibodies, ACPA) are undetectable.*

**Key words**

MICA, joint inflammation, NKG2D ligands

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## Introduction

NKG2D ligands (NKG2DLs) are important "danger-signalling" molecules in inflammatory settings. They are stress-inducible molecules, non-conventional MHC class I molecules, upregulated under various pathological conditions, such as cancer, microbial infections and inflammation (1). NKG2DLs are a family of proteins capable of engaging the NKG2D receptor, thereby activating cytotoxic lymphocytes (essentially NK and $CD8^+$ T cells) and inducing cytokine secretion (1). In man, NKG2DLs are encoded by two groups of genes: the MHC class I chain-related genes A and B (*MICA* and *MICB*) and the UL16-binding proteins (also known as retinoic acid early transcripts) ULBP1-6 genes. In mice, which do not harbour orthologous genes to *MICA* or *MICB*, three set of genes encode NKG2DLs: *Rae-1*, *H60* and *Mult-1*. Numerous studies have reported the importance of the NKG2D-NKG2DLs in tumour immunosurveillance, leading to the development of novel therapies against cancer (2). With regards to rheumatic diseases, the role of NKG2D signalling and NK cells responses in pathogenesis appears less well characterised. Interestingly, nucleotide variants in genes encoding some of these ligands, as well as the NKG2D receptor, have been associated to rheumatic diseases, like rheumatoid arthritis (RA), psoriatic arthritis (PsA) (3) or Sjögren's disease (4). MICA and MICB expression by human RA synoviocytes (RASF) has been described (5), but the underlying mechanism is still poorly described, pointing to the necessity to evaluate NKG2DLs expression in various inflammatory environments, and not only RA, in order to obtain a comprehensive vision of the role of these molecules in joint diseases.

In the present translational study, we aimed to provide a better description of the expression of NKG2DLs in various arthropathies. For this, we performed soluble MICA (sMICA) quantification by ELISA in the synovial fluid of patients affected by joint diseases. We also measured by RT-qPCR the expression levels of the genes encoding NKG2D and its ligands in several mouse models of acute and chronic joint inflammation. Our results with human samples reveal increased MICA expression in the synovial fluid of RA patients, even those in which ACPAs are undetectable and our investigations in mice point to disease-specific transcriptional control of NKG2DL. Altogether, our data suggest that, depending on the environment and its origins, inflammation mediates complex NKG2DLs / NKG2D interactions.

## Methods

*Human samples*

A total of 117 patients with various joint diseases from Strasbourg and Padua University hospitals were selected. The clinical characteristics of RA patients are given in Supplementary Table S1. All patients gave their written informed consent and the research protocol was approved by relevant institutional review boards. For 22 patients (10 OA, 5 RA, 3 REA, 2 CPP, 1 PSOA, 1 Gout), blood and synovial fluid was collected the same day. Sera from RA, OA and PSOA patients were collected and stored at the Medical University of Vienna (Austria).

*Mouse models*

Mouse models for rheumatic diseases have previously been described: Acute urate crystal inflammation in (6); Serum transfer arthritis (STA) and collagen-induced arthritis (CIA) (7). The collagenase-induced osteoarthritis model (CIOA) was described in (8). All procedures were approved by Ethic Committees on Animal Experimentation (approval CEEA-LR-10041 for the OA model and 2018083014133041 for the others) and are available upon request.

*Quantification of soluble MICA (sMICA)*

sMICA quantification was performed as described in (4). Briefly, the protocol is based on a sandwich ELISA using two different capture and one detection antibodies which recognise MICA without any allele specificity. Synovial fluid or serum was diluted 10x in PBS and all samples were tested in duplicates.

*Quantification of cytokines*

IL-1β (ref. no. 88-7261-22), IL-6 (ref.

no. 88-7066-22) and IL-8 (ref. no. 88-8086-88) were quantified using dedicated ELISA according to the Manufacturer's recommendations (eBioscience, San Diego, CA, USA).

*Real-time quantitative PCR (RT-qPCR)*

Total RNA was prepared from joints (dissected hips for the CIOA model, from hindpaws for all other models) as described in (6) and reverse transcribed using the cDNA synthesis kit (iScript ready-to-use cDNA supermix, Biorad, ref n° 4106228). Real-time quantitative RT-qPCR was performed in a total volume of 20µL using the Sso-advanced universal SYBR-Green supermix (Biorad, ref. no. 10000076382) and gene-specific primers (listed in Suppl. Table S2). After a denaturing step at 95°C for 30 seconds, 40 cycles were performed (95°C for 5s and 60°C for 20s) using a Rotor-Gene 6000 real-time PCR machine (Corbett Life Science). Results were obtained using the SDS Software (Perkin Elmer). Melting-curve analysis was performed to assess the specificity of PCR products. Relative expression was calculated using the comparative threshold cycle (Ct) method. The Ct of the gene of interest was adjusted to the average Ct of three housekeeping genes (*Hprt*, *Actin* and *Gapdh*) to obtain a ΔCt. Then, the values were normalised to the control group for each experiment and expressed as $2^{-\Delta\Delta Ct}$. The sequence of the primers is available upon request.

*Statistical analysis*

Following normality tests (Kolmogorov-Smirnov and Shapiro-Wilk), data were analysed with a Mann-Whitney test or unpaired t-test (two-tailed unpaired) to compare two independent groups and non-parametric (Spearman) test for correlation analysis. Statistics were calculated with GraphPad 5.01 software. A probability ($p$) value of <0.05 was considered significant. *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001.

## Results

We first quantified soluble MICA (sMICA) in the synovial fluid isolated from a cohort of 117 patients, among which 17 calcium pyrophosphate (CPP) crystal arthritis, 12 urate crystal arthritis (Gout), 8 reactive arthritis (REA), 13 psoriatic arthritis (PSOA), 22 rheumatoid arthritis (RA) and 45 osteoarthritis (OA). sMICA was quantified with an in-house developed sandwich ELISA using two antibodies for capture and a third for detection. This assay, which did not exhibit any MICA allelic specificity (4), showed that sMICA appears markedly increased in the synovial fluid (SF) of RA patients (Fig. 1A, 502.3±256.1 pg/ml) as compared to any of the other arthropathies tested. The lowest level of sMICA was detected in OA patients (144.7±58.03 pg/ml; $p$<0.0001). IL-1β, IL-6 and IL-8 levels are also increased in the synovial fluid of RA patients (Fig. 1B-D), although not always reaching statistical significance when compared to other arthropathies. For instance, IL-1β and IL-8 levels are comparable between gout and RA patients ($p$=0.2273 and $p$=0.1653 respectively). Similarly, IL-6 is produced in high amounts in crystalopathies caused by calcium phosphate (CPP) or urate deposits (gout), as it is the case in RA ($p$=0.2076 and $p$=0.5283). Correlation analyses showed that while sMICA quantity in the SF appears independent from IL-1β levels (Fig. 1E, $p$=0.1802), a significant correlation with IL-6 ($p$=0.0078) and IL-8 ($p$=0.0051) is evidenced (Fig. 1F-G). This indicates that *MICA* expression relies on NF-κB-dependent signalling like *Il-6* and *Il-8* genes (9). Furthermore, when we stratified RA patients according to the presence (in low or high amounts) or absence of rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPAs), we noted that sMICA expression remained unmodified (Fig. 1 H-I). These data suggest that the detection of high levels of sMICA in the synovial fluid could represent a specific biomarker of RA, which might be of particular interest in ACPA-negative subjects for which the diagnostic can be tricky. Of note, sMICA levels in the SF also appeared to be similar in untreated patients *versus* treated ones (Fig. 1-J), likely reflecting a similar inflammatory crisis that justified the synovial biopsy in all these patients. We next quantified sMICA in the serum of a cohort of RA (n=99), OA (n=101) and PSOA (n=49) patients. As seen in Fig. 2A, no difference could be detected between arthritic diseases with regards to sMICA blood levels, which appeared much lower compared to those quantified in the SF, although a significant ($p$=0.0149) correlation between paired blood/SF sMICA amount on a subset of 22 patients suffering various arthropathies could be shown (Fig. 2B). Therefore, in contrast to SF, serum appeared not to be a reliable fluid to quantify sMICA in order to discriminate RA from OA or PSOA.

To gain a global view on the possible involvement of all NKG2DLs in joint diseases, we used various mouse experimental models mimicking four different arthritides: (1) subcutaneous injection of monosodium urate (MSU) crystals to induces an acute inflammation resembling a gout crisis (6), (2) serum transfer arthritis (STA) which is an established model of acute arthritis and the K/BxN, (3) collagen-induced arthritis (CIA) models to reproduce rheumatoid arthritis, and (4) the collagenase-induced osteoarthritis (CIOA) to drive defects similar to those seen in human osteoarthritis. Given the variety of genetic backgrounds of these models (C57Bl/6, DBA/1, composite C57Bl/6 and NOD1) and the important homology between murine NKG2DLs, designing primers to specifically amplify the corresponding genes was challenging. We identified and validated 5 pairs of primers respectively amplifying *H60a* (a pseudogene in C57Bl/6 but not in NOD1 and DBA/1 mice), *H60b*, *H60c*, *Rae1*ε and *Rae1*δ simultaneously, and *Mult1*. The expression of the *Klrk1* gene encoding murine NKG2D was also quantified. As shown in Fig. 3, *H60a* expression is induced 47 days following collagen immunisation in the CIA model (Fig. 3A), *H60b* transcription increases upon arthritogenic serum transfer (Fig. 3B) and *H60c* expression decreases in the acute models of urate inflammation (gout) and arthritis (Fig. 3C). The transcription of the second family of ligands, encoded by the *Rae1*ε and Rae1δ genes which are amplified by the same primers pair is induced in the acute inflammatory models (MSU and STA), as well as in the chronic

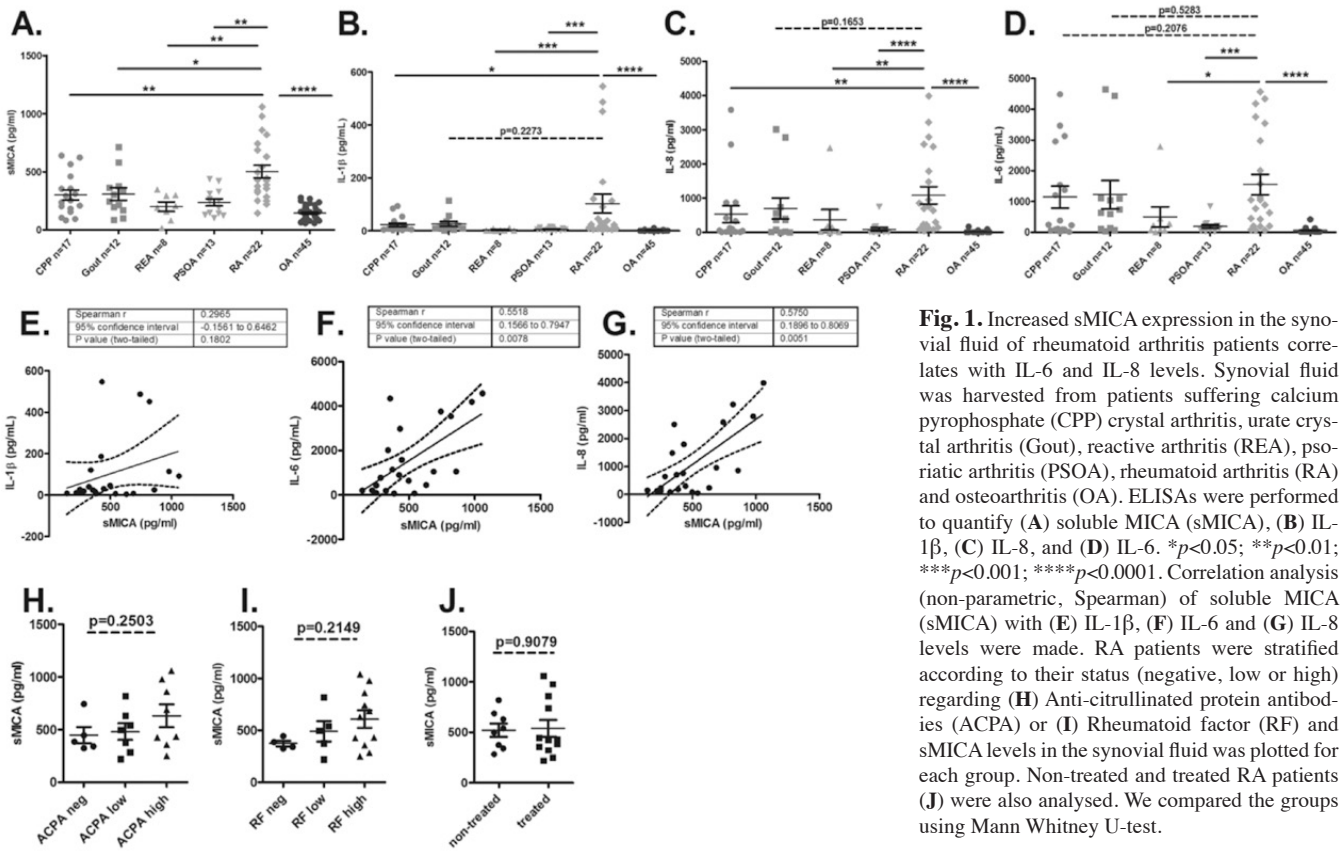


**Fig. 1.** Increased sMICA expression in the synovial fluid of rheumatoid arthritis patients correlates with IL-6 and IL-8 levels. Synovial fluid was harvested from patients suffering calcium pyrophosphate (CPP) crystal arthritis, urate crystal arthritis (Gout), reactive arthritis (REA), psoriatic arthritis (PSOA), rheumatoid arthritis (RA) and osteoarthritis (OA). ELISAs were performed to quantify (**A**) soluble MICA (sMICA), (**B**) IL-1β, (**C**) IL-8, and (**D**) IL-6. **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001. Correlation analysis (non-parametric, Spearman) of soluble MICA (sMICA) with (**E**) IL-1β, (**F**) IL-6 and (**G**) IL-8 levels were made. RA patients were stratified according to their status (negative, low or high) regarding (**H**) Anti-citrullinated protein antibodies (ACPA) or (**I**) Rheumatoid factor (RF) and sMICA levels in the synovial fluid was plotted for each group. Non-treated and treated RA patients (**J**) were also analysed. We compared the groups using Mann Whitney U-test.

CIA model (Fig. 3D). Expression of the third family, *Mult1*, is induced in both acute (STA) and chronic (K/BxN and CIA) models of arthritis (Fig. 3E). Several information can be extracted from these data: (i) while *Rae1* genes appear to be induced in all inflammatory arthritides (gout and RA models, acute and chronic), *Mult1* overexpression seems more specific to RA models; (ii) *H60b and H60c* genes are differentially regulated in the acute STA model of RA, suggesting opposite functions for these NKG2DLs in this inflammatory setting; (iii) strikingly, none of the genes encoding NKG2DLs are induced in the osteoarthritis model, which supports human observations indicating a completely different etiology for this disease (in which cartilage damage resulting from injuries induces subsequent inflammation) compared to other inflammatory arthritides such as RA (in which inflammation drives articular damages) (10). Finally, we observed reduced *Klrk1* transcription upon serum transfer (STA) and in the chronic K/BxN arthritis models (Fig.3F), which might result from continuous exposure

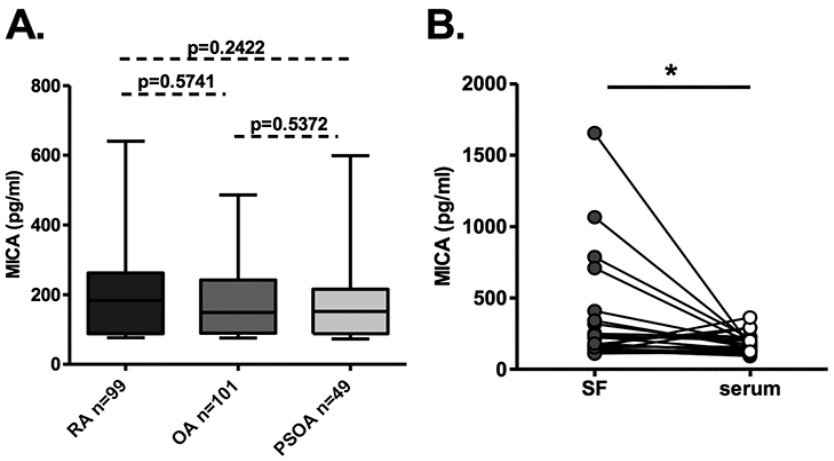


**Fig. 2.** Quantification of soluble MICA in the serum does not discriminate RA, OA and PSOA patients.
**A**: Soluble MICA (sMICA) was quantified by ELISA in the serum of rheumatoid arthritis (RA), osteoarthritis (OA) and psoriatic arthritis (PSOA) patients.
**B**: For 22 arthritis patients, sMICA was quantified in the serum and the synovial fluid (SF) harvested the same day. **p*<0.05.

of the receptor to its ligands (RAE1ε and δ, MULT1 whose corresponding transcripts are induced in these conditions), as seen for sMICA and cell surface NKG2D in human NK cells (11).

## Discussion

Production of soluble NKG2DL has been essentially studied in the context of tumour cells as an evasion mechanism from immune cells (NK and T cells) cytotoxicity. In joint diseases, NKG2DL were mainly investigated in RA. In line with previous reports (3), our data reveal increased sMICA expression in the synovial fluid (SF) of RA patients. As pointed by others, *MICA* upregulation by fibroblast-like

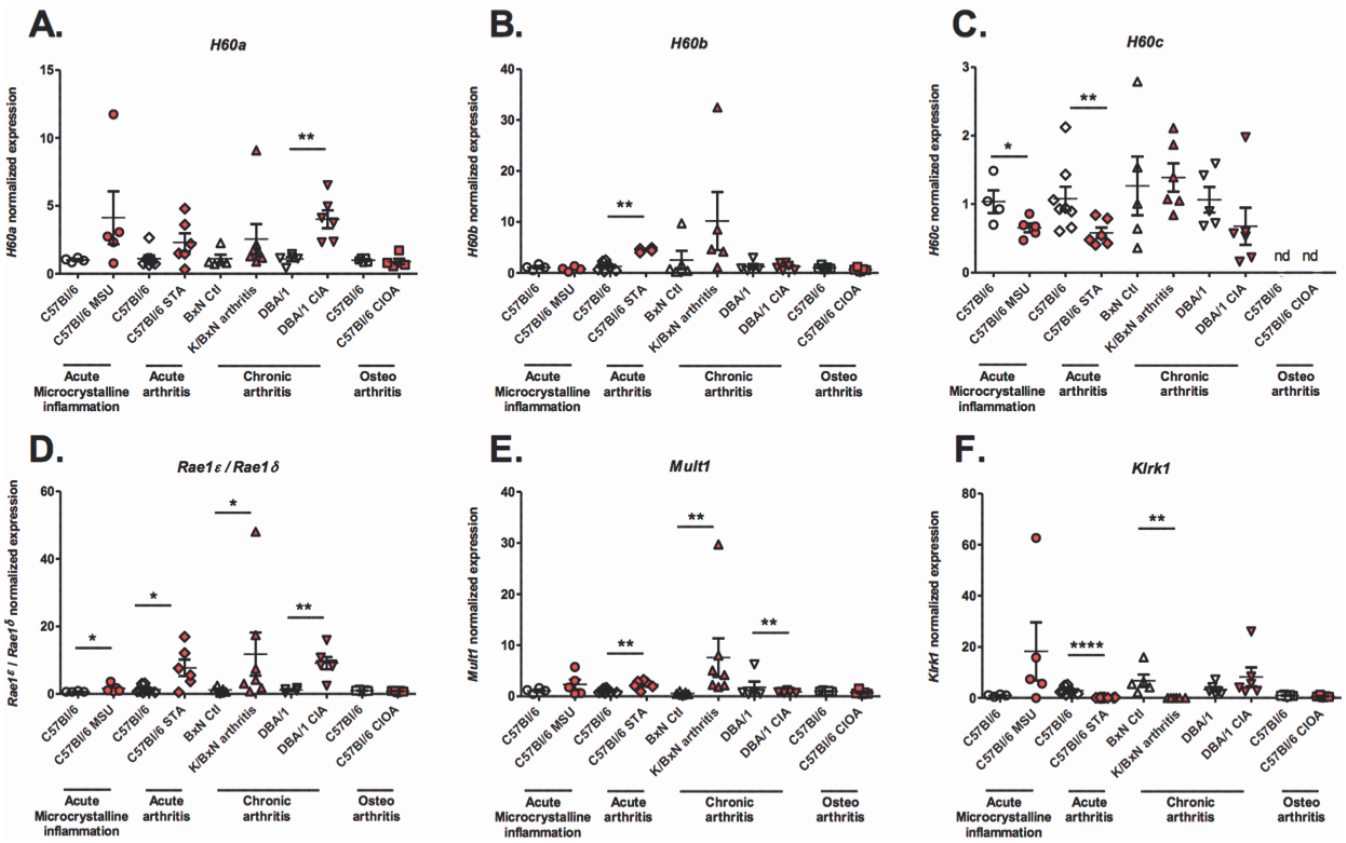


**Fig. 3.** NKG2D ligands and receptor gene expression in various mouse models of joint inflammation. Mice (n=4 to 8) subjected to monosodium urate (MSU) inflammation, serum-transfer arthritis (STA), collagen-induced arthritis (CIA), collagenase-induced osteoarthritis (CIOA) or mice exhibiting spontaneous and chronic arthritis (K/BxN) and the corresponding controls were used. RNA from dissected joint was purified, reverse transcribed and quantified. RNAs corresponding to (**A**) *H60a*, (**B**) *H60b*, (**C**) *H60c*, (**D**) *Rae1*ε/δ, (**E**) *Mult1* and (**F**) *Klrk1* genes were normalised to housekeeping genes to obtain a ΔCt which was adjusted to that of the controls using the $2^{-\Delta\Delta Ct}$ method. *$p<0.05$; **$p<0.01$. nd: not detectable.

synoviocytes (FLS) of RA patients has been suggested as an important event in the perpetuation of the disease. Increased sMICA detection in the synovial fluid of RA patients might result from the accumulation of DNA damage associated with FLS proliferation (12), a hallmark of RA pathogenesis. Furthermore, FLS from RA patients also express matrix metalloproteases (MMPs) (13), which, in addition to participating to their invasive properties, could also be responsible to membrane-bound MICA shedding and sMICA production in the synovial fluid. Interestingly, Toll-like receptor (TLRs) stimulation by ligands such as LPS can also trigger *MICA* expression by monocytes and the resulting activation of NK cells and subsequent interferon-γ secretion might fuel inflammatory responses (14). This observation indicates that *MICA* expression could result from danger-sensing during other arthropathies and independently from synovial cells proliferation. Our quantification of sMICA in the synovial fluid harvested from patients suffering various crystalopathies (resulting from urate or phosphate deposits), psoriatic arthritis or reactive arthritis does not support this possibility, although we could not directly evaluate *MICA* gene expression at the mRNA or membrane-bound levels from synovial tissues. Indeed, it is conceivable that reduced sMICA could also result from the absence of proteases responsible for MICA shedding. Furthermore, other NKG2DLs could also be produced in the synovium of arthritides with different etiologies and contribute to the maintenance of an inflammatory setting. However, our results also appear contradictory to others (15) who detected increased MICA expression in the serum of RA patients. Such discrepancy might result from differences in the specificities of the antibodies that were used in the ELISA assays as well as in the genetic characteristics of the populations that were investigated. Next, we quantified transcripts encoding H60, Rae1 and Mult1 family members in various joint inflammatory mouse models, mimicking acute or chronic diseases, either induced or spontaneous. Our work revealed that *Rae1*ε/δ expression is induced in all models of joint inflammation, except the CIOA, pointing to an additional etiological difference between OA and other arthritides. Interestingly, *Rae1*ε/δ appeared also upregulated in joints harvested from MSU crystals-injected mice, suggesting that cellular damage and /or activation following urate crystal-induced inflammation might respond to NKG2D / NKG2DLs cross talk, further supporting a regulatory role for NK or NKT cells in gout (16). Furthermore, down regulation of *H60c* expression in the same conditions suggests the existence of a balanced regulation of NKG2DLs expression in gout.

## Conclusions

Altogether, our data suggest that sMICA quantification in the synovial fluid could be an interesting biomarker to confirm a diagnosis of RA, particularly when ACPAs are undetectable in a significant proportion of RA patients. This will need to be confirmed in future prospective studies. They also reveal a complex and differential transcriptional regulation of the genes encoding murine NKG2DLs under various inflammatory settings. Our observations indicate that, in addition to MICA, additional ligands should be quantified in the synovial fluid of arthritic patients with joint diseases of different aetiologies. This would enhance our understanding of the complex cellular interactions involving NK or NKT cells in the inflammatory microenvironment in these diseases and potentially reveal novel therapeutic opportunities.

## Significance and innovations

- Clinically, this work suggests that the quantification of soluble MICA in the synovial biopsy performed in a patient with joint inflammation of undetermined origin might, in conjunction with additional markers, help considering the possibility of rheumatoid arthritis. Such information could accelerate the prescription of appropriate therapeutic measures.
- Fundamentally, this study indicates that transcriptional regulation of genes encoding Nkg2d ligands might reflect specific inflammatory settings.

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