

# Increased intra-articular granzyme M may trigger local IFN- $\lambda$ 1/IL-29 response in rheumatoid arthritis

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## Abstract

### Objective

Granzymes are serine proteases involved in eliminating tumour cells and virally infected cells. In addition, extracellular granzyme levels are elevated in inflammatory conditions, including several types of infection and autoimmune diseases, such as rheumatoid arthritis (RA). While GrA and GrB have been associated with RA, a role for the other three granzymes (GrH, GrK, and GrM) in this disease remains unclear. Here, we aimed to investigate the presence and role of GrM and GrK in serum and synovial fluid of patients with RA, psoriatic arthritis, and osteoarthritis.

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### Methods

Granzyme levels were determined in serum, synovial fluid, peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMCs) of RA patients and relevant control groups. In addition, the link between GrM and inflammatory cytokines in synovial fluid was investigated.

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### Results

Serum GrM and GrK levels were not affected in RA. GrM, but not GrK, levels were elevated in synovial fluid of RA patients. GrM was mainly expressed by cytotoxic lymphocytes in SFMCs with a similar expression pattern as compared with PBMCs. Intra-articular GrM expression correlated with IL-25, IL-29, XCL1, and TNF $\alpha$  levels. Intriguingly, purified GrM triggered the release of IL-29 (IFN- $\lambda$ 1) from human fibroblasts *in vitro*.

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### Conclusion

These data indicate that GrM levels are increased in RA synovial fluid and that GrM can stimulate proinflammatory IL-29 release from fibroblasts, suggesting a role of GrM in the pathogenesis of RA.

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### Key words

rheumatoid arthritis, granzyme, cytokines, inflammation

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## Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that is characterised by inflammation and hyperplasia of the synovial lining of the joints (1). Inflamed synovial tissue and inflammatory cells in the synovial fluid induce destruction of cartilage and bone. Despite an enormous progress in understanding the inflammatory processes, the pathogenesis is still not fully understood. In RA, the extensive lymphocyte infiltration in the inflamed joint contributes to immunopathology by the production of inflammatory and cytotoxic mediators (1, 2). Detailed analysis of cytokine expression in RA has provided remarkable insight into disease pathogenesis (3, 4). The critical role of IL-1, IL-6, and TNF- $\alpha$  has been demonstrated most extensively, by successful clinical targeting of these cytokines in RA (5, 6). Recent insights suggest that members of the granzyme family could play a significant role in RA immunopathology.

Granzymes are serine proteases produced by cytotoxic lymphocytes (cytotoxic T cells,  $\gamma\delta$  T cells, natural killer (NK) cells and NKT cells). There are 5 human granzymes: granzyme A (GrA), GrB, GrH, GrK, and GrM, which induce apoptosis in tumour cells and virally infected cells (7, 8). Soluble granzyme levels are elevated in blood of patients with various infections and autoimmune diseases (9). These extracellular granzymes can trigger proinflammatory cytokine release and therefore granzymes are thought to perform additional functions in inflammatory disease (10). Extracellular GrK and GrA are able to induce proinflammatory cytokine release (e.g. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8) from human cells, while GrB is thought to have more effect on cytokine cleavage and activation than on cytokine release (11, 12). The contribution to cytokine release and/or activation may thus aggravate inflammation in RA. Cleavage of extracellular matrix components by GrA and GrB is also thought to contribute to tissue destruction in RA (9). Elevated levels of GrA and GrB are described in RA synovial fluid and plasma compared to controls (9). Increased GrB expression is ob-

served in RA synovial tissue, but not in control osteoarthritis (OA) tissue (13). In the synovial tissues of RA joints, GrB expression was strongly observed in the lining layers where mostly macrophages and some lymphocytes are located (13). Next to this, GrB also contributes to apoptosis of chondrocytes *in vitro*, and single-nucleotide polymorphisms in the GrB gene influence the rate of joint destruction in RA (14, 15). In animal studies, GrA contributes to joint destruction in RA, in part by promoting osteoclast differentiation (16). To date, the role of extracellular granzymes other than GrA and GrB in RA remains unclear. In the present study, we aimed to investigate the levels of GrK and GrM in serum and synovial fluid from RA patients, psoriatic arthritis (PsA) patients, and OA patients. In addition, we addressed the cellular expression of GrM in synovial fluid-derived mononuclear cells (SFMCS) of patients with RA and the relation between GrM and proinflammatory cytokines.

## Methods

### Human serum and synovial fluid samples

Serum and synovial samples from patients were obtained at the University Medical Center (UMC) Utrecht. Synovial fluid from OA patients is used as a non-inflammatory control group for inflammatory arthritic conditions like RA and PsA. PsA is an inflammatory disease control group to RA, to reveal 'arthritis' specificity. The use of patient samples was approved by the ethics committee of the UMC Utrecht (protocol number 05-192/O) and all patients gave their written informed consent. RA patients were classified according to the American College of Rheumatology criteria (17). Synovial samples and serum samples were not matched. Clinical data on RA, PsA, and OA patients are presented in Table I. The disease duration of all patients was variable (1–51 years). Serum samples from healthy volunteers (age and gender matched) were obtained from the UMC Utrecht mini-donor service. All subjects had no recorded history of recent infection. All donors had given written informed consent.

Competing interests: none declared.

*Human SFMC and PBMC isolation*

Paired peripheral blood mononuclear cells (PBMCs) and SFMCs were collected from RA patients (n=9) as described previously (18). SFMCs were from knee joints. Paired PBMCs and SFMCs were aliquoted and cryopreserved on the same day after density gradient isolation and thawed on the day of the experiments using standardised protocols.

*Granzyme and cytokine measurements in serum and synovial fluid samples*

The serum of RA (n=30), PsA (n=17) patients and HC (n=10), and the synovial fluid of RA (n=37), PsA (n=37) and OA (n=10) were preventively pretreated with heteroblock (Omega Biologicals) (150 µg/ml in undiluted samples), for 1h at room temperature with shaking. GrM and GrK levels in serum and synovial fluid samples were determined using ELISA (Uscn Life Sciences). The lower detection limit for GrM is 3.9 pg/ml and for GrK is 7.8 pg/ml. ELISAs were performed according to the manufacturer's instructions. Cytokines (TNF-α, IL-25, IL-29, and XCL1) from synovial fluid were measured using a luminex assay as described previously (19) on a Luminex FlexMap 3D (BioRad) with xPonent 4.2 software. Other cytokines that were measured in synovial fluid by luminex include IL-1β, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-16, IL-17, IL-21, IL-22, IL-23, IL-31, IL-37, TNFβ, IFNα, IFNγ, MIF, TSLP, I-309, MCP-1, MIP-1α, MIP-1β, MCP-3, MCP-2, MCP-4, TARC, MIP-3β, MIP-3α, MDC, MPIF-1, TECK, C-TACK, PF4, IL-8, MIG, IP-10, I-TAC, SDF-1α, BLC, BRAK, PSOX, GM-CSF, HGF, MMP-1, sIL-2Rα, and C5a. Lower detection limits (pg/ml) for TNF-α, IL-25, IL-29 and XCL1 measured by luminex assay are 0.7, 49, 6.3, and 5.1, respectively. Data were analysed using BioPlex Manager 6.1.1 (BioRad).

*Granzyme M FACS staining*

Matched PBMCs and SFMCs were incubated with cell-surface markers prior to determine GrM protein levels: CD3-PerCP-Cy5.5 (1:25; UCHT1; Biolegend), CD4-PB (1:50; RPA-T4;

Biolegend), CD8-PE (2:25; RPA-T8; Biolegend), γδTCR-APC (4:25; B1; BD), CD16-V500 (1:50; 3G8; BD), CD14-BV785 (1:100; M5E2; Biolegend), CD56-PE-Cy7 (1:25; B159; BD) and CD19-BV605 (1:50; SJ25C1; BD). Cells were subsequently permeabilised for intracellular GrM staining. After fixation and permeabilisation, cells were incubated with Alexa Fluor 488-conjugated anti-GrM (1:2000; clone 4B2G4) as described previously (8, 20). Dead cells were outgated with fixable viability dye eFluor780 (1:1000; eBioscience). Monocytes were identified based on characteristic properties of the cells in the forward (FSC) and side scatter (SSC) and then gated for CD14<sup>+</sup> monocytes. Within the lymphocyte gate based on FSC/SSC, the T cells were gated on CD3<sup>+</sup> and then divided in CD4<sup>+</sup> or CD8<sup>+</sup>. NK cells were gated as CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> and NKT cells were gated as CD3<sup>+</sup>CD56<sup>+</sup>. γδTCR cells were gated as CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>γδTCR<sup>+</sup>. B cells were characterised as CD3<sup>-</sup>CD19<sup>+</sup>. Within these populations, the percentage of GrM<sup>+</sup> cells and geometric mean of GrM expression was defined. Fluorescence-minus-one (FMO) control stains were included to determine background levels of staining. Geometric mean of GrM expression in each population was corrected by that of FMO control. After staining, cells were washed and immediately analysed by flow cytometry (FACS Canto II; BD Bioscience). The data were analysed with FACS DIVA software.

*Stimulation of human fibroblasts by granzyme M*

Human foreskin fibroblasts (primary fibroblasts) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells (50,000/well) were seeded in 48-wells plates and stimulated o/n under standard incubator conditions (37°C, 5% CO<sub>2</sub>, relative humidity 95%), with 300 nM GrM or 300 nM GrM-SA (catalytically inactive control). GrM(-SA) was produced and purified [endotoxin-free, <0.5 Endotoxin Unites (EU)/ml, as determined by LAL

assay] as described previously (21, 22). After stimulation, supernatants were collected and stored at -80°C until measurement with luminex.

*Statistics*

Comparisons between groups were made using Mann-Whitney U-test when comparing 2 groups or Kruskal-Wallis with post-hoc Dunn's when comparing more than 2 groups. Paired analysis between groups were made using Wilcoxon matched-pairs signed rank test. Correlations between parameters were tested using the Spearman rank correlation. All tests were conducted two-sided with an alpha level set at 0.05. Statistical analyses were performed using graphpad prism (v. 6).

**Results***GrK and GrM levels are not elevated in RA serum*

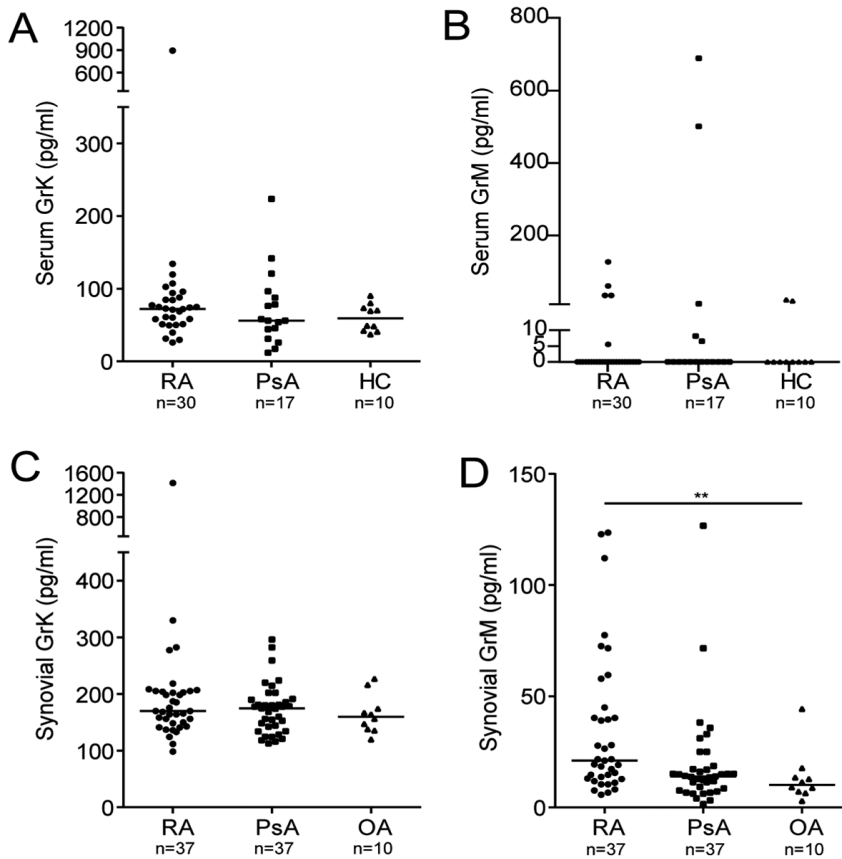
GrK and GrM levels were determined in serum from RA patients (n=30), PsA patients (n=17), and healthy controls (n=10). No significant differences were found in either serum GrK or serum GrM levels in RA patients or PsA patients when compared to healthy controls (Fig. 1A-B) nor was there a relation with clinical phenotype of disease.

*GrM but not GrK levels are elevated in RA synovial fluid*

GrK and GrM levels in synovial fluid from RA (n=37), PsA (n=37), and OA (n=10, control) patients were determined. GrK levels in RA or PsA groups were not elevated compared to levels in OA patients (Fig. 1C). Interestingly, however, GrM levels were significantly elevated in synovial fluid from RA patients, compared to OA controls (Fig. 1D). No significant differences in GrM levels were found between PsA patients and OA patients ( $p=0.25$ ), and no correlation between GrM levels in synovial fluid and circulating C-reactive protein (CRP) levels or erythrocyte sedimentation rate (ESR) was found in RA patients (Table I).

*Intracellular GrM is expressed in synovial fluid-derived immune cells*

To address the question whether the GrM present in the synovial fluid can



**Fig. 1.** GrK and GrM levels in RA, PsA and OA serum and synovial fluid. Serum levels of GrK (A) and GrM (B) in RA (n=30) or PsA (n=17) patients or healthy controls (n=10) and synovial fluid levels of GrK (C) and GrM (D) in RA (n=37), PsA (n=37), and OA (n=10) patients were measured using ELISA. Median granzyme levels are depicted and statistical analysis was performed using the Mann-Whitney U-test. \*\**p*<0.01 compared to OA control.

**Table I.** Clinical and demographic patient characteristics.

Data	RA		PsA		OA
	Serum (n=30)	SF (n=37)	Serum (n=17)	SF (n=37)	SF (n=10)
Age (average + range)	61.1 (35-84)	54 (19-83)	50.8 (29-70)	46 (31-73)	63 (53-78)
Gender (m/f/N.R.)	8/22	12/25	7/10	24/13	3/6/1
ESR (mm/1st hour)*	14.3 ± 8.4	42.6 ± 32.1	N.R.	18.3 ± 17.2	11.3 ± 8.6
CRP (mg/L)**	3.5 ± 1	42.2 ± 34.1	6.5 ± 7.4	13.4 ± 13.4	6.3 ± 1.2
Immunosuppressive drugs	N.R.	34/37	16/17	37/37	8/10

SF: synovial fluid; NR: not registered.

For serum measurements serum from healthy volunteers was used as a control.

\*Erythrocyte sedimentation rate (ESR) was available in the following patients: RA serum 28/30; PsA serum 0/17; RA synovial fluid 35/37; PsA synovial fluid 37/37 and OA synovial fluid 4/10 patients.

\*\*C-reactive protein (CRP) levels were available in the following patients: RA serum 4/30; PsA serum 4/17; RA synovial fluid 16/37; PsA synovial fluid 14/37 and OA synovial fluid 3/10 patients.

be locally produced, we measured GrM expression in SFMCs of RA patients. Highest intracellular GrM expression was observed in cytotoxic cell subsets (CD8<sup>+</sup> T cells, NKT,  $\gamma\delta$ T, and NK cells) in both (paired) SFMCs and PBMCs (Fig. 2A-B). Significantly

elevated GrM expression was found in NK, NKT and  $\gamma\delta$ T cell subsets of PBMCs compared to these cell subsets in SFMCs. Moderate to lower GrM expression in both SFMC and PBMC subsets were found in CD4<sup>+</sup> T cells, B cells and CD14<sup>+</sup> monocytes. The percentage

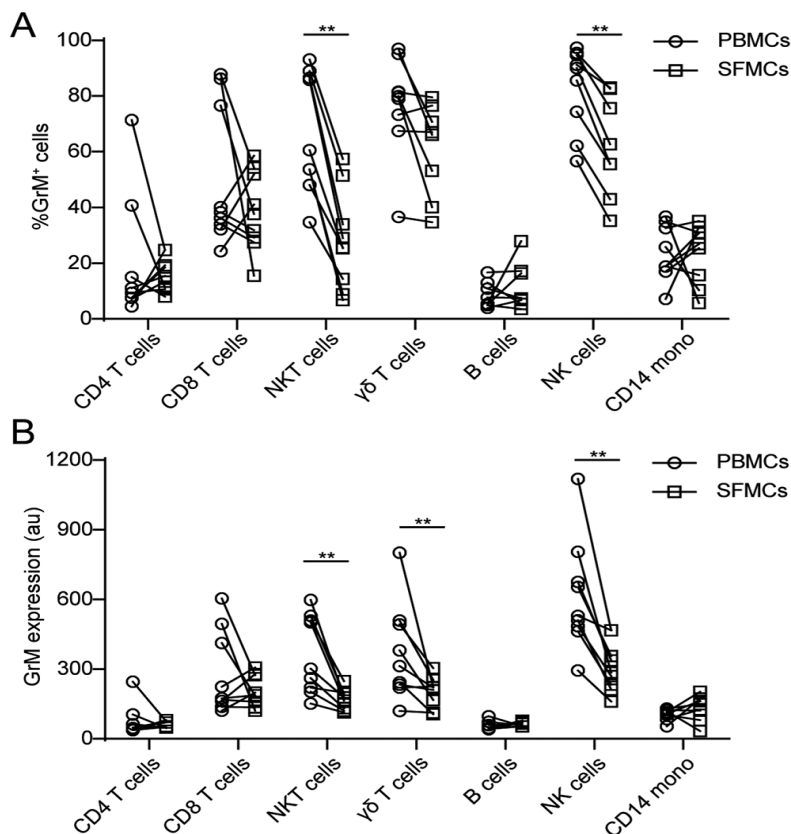
of GrM positive cells in synovial fluid-derived NK cells and NKT cells was significantly decreased as compared to their circulating counterparts. The expression pattern of CD4<sup>+</sup> and CD8<sup>+</sup> T cells,  $\gamma\delta$ T cells, B cells, and CD14<sup>+</sup> monocyte cell subsets was not different between synovial fluid and blood (Fig. 2A). We conclude that several cytotoxic cell subsets contribute to GrM production in SFMCs, suggesting that GrM can potentially be locally produced in the synovial tissue.

*GrM levels in synovial fluid correlate with local cytokine levels*

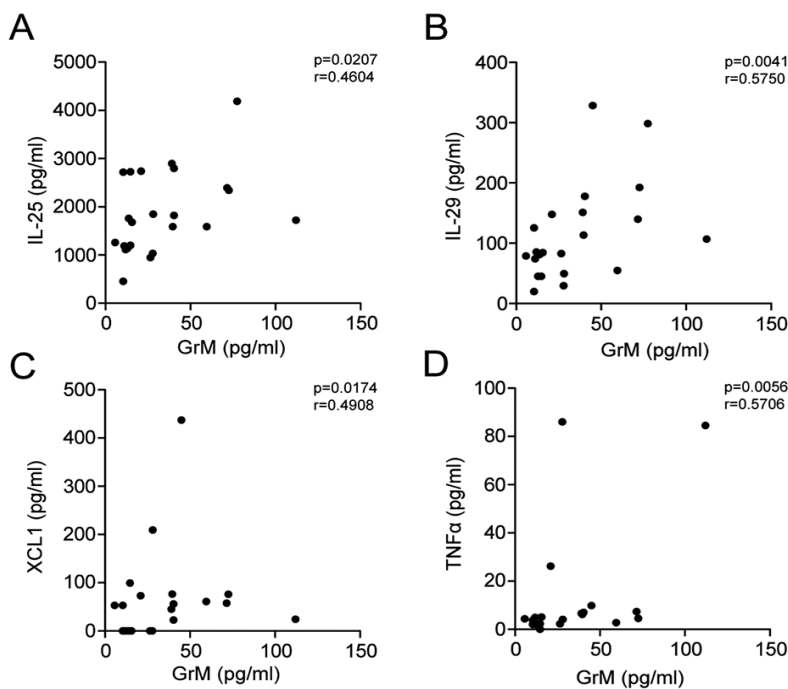
Previously, it has been demonstrated that GrM induces cytokine production (23). To reveal a possible link between GrM and local cytokine levels in the synovial fluid, GrM levels from synovial fluid were correlated to a panel of proinflammatory cytokines (n=52). Statistical analysis revealed that synovial fluid concentrations of IL-29, TNF- $\alpha$ , IL-25, and XCL1, were significantly correlated to GrM levels (Fig. 3A-D). Although many cytokines were increased in the synovial fluid of RA patients (data not shown), statistically significant correlations between all other cytokines and GrM were not observed.

*GrM potentiates IL-29 release from human fibroblasts*

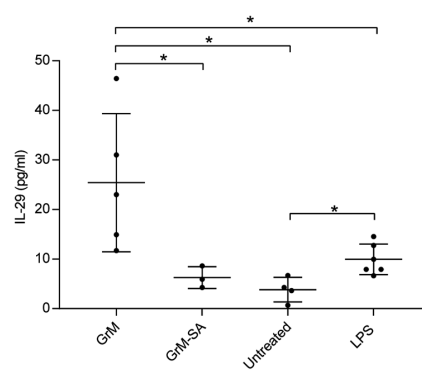
Since GrM levels in the synovial fluid correlate to levels of proinflammatory cytokines, we addressed the question whether GrM can trigger cytokine release from synovial fluid-derived SFMCs. Following stimulation of SFMCs (and PBMCs) with isolated GrM, no induction of TNF- $\alpha$ , XCL1, IL-25, or IL-29 was observed (data not shown). Since the synovium also includes other cell types than SFMCs, we next tested the cytokine-inducing effect of GrM on human fibroblasts. Whereas TNF- $\alpha$ , XCL1, and IL-25 were not detectable in the medium following GrM treatment, we found an increase of IL-29 following incubation with GrM (Fig. 4). Interestingly, this effect was not found with a GrM mutant (GrM-SA), in which the serine in the catalytic centre has been replaced



**Fig. 2.** Granzyme M expression in mononuclear cell subsets from peripheral blood and synovial fluid. GrM-positive cells (A) and GrM expression (au, arbitrary units) (B) were investigated by FACS analysis in indicated paired cell subsets of PBMCs (open circles) and SFMCs (open squares) from a random selection of RA patients (n=9). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test. \*\*  $p < 0.01$  compared to PBMC cell subset.



**Fig. 3.** GrM levels in RA synovial fluid correlate with proinflammatory cytokines. GrM levels in synovial fluid were correlated to cytokines expressed in the synovial fluid of a random selection of RA patients (measured by luminex). Spearman rank correlation values are indicated.



**Fig. 4.** Isolated GrM triggers IL-29 release from fibroblasts. Fibroblasts were incubated with isolated GrM (n=5), catalytically inactive GrM-SA (n=5), LPS (2 EU/ml) (n=6), or left untreated (n=6) for 22h and cytokine release was measured by luminex. Only values above detection limit are shown (detection limit: 0.67 pg/ml). Statistical analysis was performed using the Mann-Whitney U-test. \* $p < 0.05$ .

by Alanine (catalytically dead GrM variant) (Fig. 4). This suggests that the potential of GrM to release IL-29 from human fibroblasts is fully dependent on the catalytic activity of the protease.

**Discussion**

Here we report that GrM is elevated in the synovial fluid of RA patients, but not in the blood circulation (Fig. 1). In contrast, GrK is neither upregulated in serum nor in synovial fluid of RA, PsA, or controls. Previously, it has been demonstrated that GrA and GrB are present in both serum and synovial fluid (9). Apparently, granzyme expression is individually regulated in RA rather than simultaneously. To determine whether the amounts of GrM present in synovial fluid can be a result of local production, GrM expression was investigated in SFMCs (Fig. 2). Indeed, synovial fluid-derived mononuclear cells show GrM expression with highest expression in cytotoxic T lymphocytes and NK cells (Fig. 2). Intriguingly, isolated GrM can trigger the release of IL-29 from fibroblasts (Fig. 4). Hence, our data point to a local role of GrM in the pathophysiology of RA by stimulating release of at least the proinflammatory cytokine IL-29, a type-III interferon cytokine that is also called IFN- $\lambda$ 1 (19, 24).

The cellular granzyme source that mediates the local increase in GrM levels remains unknown. We demonstrated

that GrM is mainly expressed by NK cells, NKT cells,  $\gamma\delta$ T cells, CD8<sup>+</sup> T cells and to a lesser amount by CD4<sup>+</sup> T cells, B cells, and CD14<sup>+</sup> monocytes in synovial RA mononuclear cells (Fig. 2). GrM protein expression (absolute expression and percentage of cell subsets) in SFMC subsets is lower in NK cells, NKT cells, and partly in  $\gamma\delta$ T cells, as compared to their paired PBMC counterparts (Fig. 2). This is compatible with previous observations that the cytotoxic potential of SFMCs in arthritis patients is diminished, *e.g.* via downregulation of granzyme gene expression (19, 25, 26). Lower GrM protein expression in SFMCs compared to PBMCs could also point to active release of the intracellular GrM pool into the synovial fluid. Local triggers (*e.g.* cytokines in synovial fluid) are likely responsible for local GrM release, since GrM-positive cytotoxic cells circulate in blood and no increase of serum GrM is observed (Fig. 1). In addition, GrM expression might be affected by clinical parameters, including disease activity, duration, and/or therapy. Finally, we cannot exclude the possibility that other cells than SFMCs in the synovium can produce and secrete GrM, such as tissue macrophages, fibroblasts, or neutrophils. Extracellular GrM, but not a catalytically dead mutant GrM variant, can induce IL-29 release from human fibroblasts in a perforin-independent manner (Fig. 4). This suggests that extracellular GrM cleaves a substrate likely on the cell surface of fibroblasts that leads to IL-29 secretion. This would be analogous to GrK that can cleave and activate the cell-surface PAR-1 receptor on fibroblasts, leading to intracellular signal transduction and IL-6 release (19, 27). Alternatively, GrM is taken up by fibroblasts via endocytosis to exert intracellular proteolytic effects to induce proinflammatory cytokines (19, 23). It remains unknown how the physiological local GrM concentration in the synovial fluid or (cell-bound) in the extracellular matrix *in vivo* relates to our *in vitro* cell culture experiments. Since heterogeneity exists between fibroblast types, it would be interesting to study the effects of GrM on IL-29 release from primary synovium-derived hu-

man fibroblasts gathered from multiple RA patients. Therefore, for the moment we can only conclude that extracellular GrM has the potential to induce IL-29 from fibroblasts *in vitro* (Fig. 4). It has been well established that IL-29 is elevated in the serum and synovial fluid of RA patients, where it contributes to RA pathogenesis (19, 24, 28). IL-29 promotes inflammation by inducing proinflammatory cytokine production (19, 29, 30). In addition, IL-29 might enhance local cartilage degradation, as recently demonstrated in OA (19, 31). Further research is required to elucidate the importance and role of the GrM-IL-29 axis in RA pathogenesis.

A limitation of this study is the relative small number of included patient samples. We cannot fully rule out that statistically significant increases of GrM and/or GrK in sera or synovial fluid of the different patient groups have been missed. Future studies with larger patient cohorts are therefore required. Nevertheless, in the present study we could detect for the first time a statistically significant increase of GrM in the synovial fluid of RA patients (Fig. 1). In conclusion, GrM and GrK levels are not changed in RA serum, whereas GrM, but not GrK, levels are increased in RA synovial fluid. Several cytotoxic cell subsets, including CD8<sup>+</sup> T cells, NKT,  $\gamma\delta$ T, and NK cells, contribute to GrM production in SFMCs. Proinflammatory cytokines IL-29, TNF- $\alpha$ , IL-25, and XCL1 correlate with GrM levels in synovial fluids, and GrM can stimulate IL-29 release from human fibroblasts. Our data point to a novel mechanism by which intra-articular GrM produced by (cytotoxic) cells may contribute to RA disease progression by locally inducing proinflammatory IL-29. This raises the possibilities that GrM may serve as a biomarker in RA and/or that targeting GrM can prevent IL-29 induction in an early disease stage in a subset of patients.

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