Rheumatoid arthritis synovial fluid shows enrichment of T-cells producing GMCSF which are polyfunctional for TNF-α and IFNγ

A. Khullar[1], V. Dhir[1], B. Saikia[2], A.K. Yadav[3], B. Leishangthem[1], C.B. Prasad[1], S. Jayaprakash[1], S. Jain[1], G.S.R.S.N.K. Naidu[1], S.K. Sharma[1], A. Sharma[1], S. Jain[1]

[1]Division of Rheumatology, Department of Internal Medicine; [2]Department of Immunopathology; [3]Department of Experimental Medicine and Biotechnology, PGIMER, Chandigarh, India.

Abstract

Objective

GMCSF+T-cells may be involved in pathogenesis of rheumatoid arthritis (RA), and polyfunctionality may be a marker of pathogenicity. Although, higher frequencies of CD4+GMCSF+ T-cells have been reported, there are no data on CD8+GMCSF+ T-cells or polyfunctionality.

Our objective was to enumerate frequencies of CD8+GMCSF+ T cells in RA blood and synovial fluid (SF), and assess their polyfunctionality, memory phenotype and cytotoxic ability.

Methods

This study included RA patients (blood samples,in some with paired synovial fluid (SF)), healthy controls (HC) (blood) and SpA patients (SF). In some RA patients' blood was sampled twice, before and 16–24 weeks after methotrexate (MTX) treatment. After mononuclear cell isolation from blood and SF, ex-vivo stimulation using PMA/Ionomycin was done, and cells were stained (surface and intracellular after permeabilisation/fixation). Subsequently, frequencies of GMCSF+CD8+ and CD4+ T-cells, polyfunctionality (TNFα, IFNγ, IL-17), phenotype (memory) and perforin/granzyme expression were assessed by flowcytometry.

Results

There was no significant difference in frequencies of GMCSF+CD8+ (3.7, 4.1%, p=0.540) or GMCSF+CD4+ T-cells (4.5, 5.2%, p=0.450) inblood of RA and HC. However, there was significant enrichment of both CD8+GMCSF+ (5.8, 3.9%, p=0.0045) and CD4+GMCSF+ (8.5, 4.5%, p=0.0008) T-cells inSF compared to blood in RA patients. Polyfunctional triple cytokine positive TNFα+IFNγ+GMCSF+CD8+T-cells (81, 36%, p=0.049) and CD4+T-cells (48, 32%, p=0.010) was also higher in SF compared to blood in RA. CD8+ T cells showed higher frequency of effector-memory phenotype and granzyme-B expression in RA-SF. On longitudinal follow-up, blood CD4+GMCSF+ T-cells significantly declined (4.6, 2.9%, p=0.0014) post-MTX.

Conclusion

We report a novel finding of enrichment of CD8+GMCSF+ in addition to CD4+GMCSF+ T-cells in RA-SF. These cells showed higher polyfunctionality for TNFα and IFNγ, and effector memory phenotype suggesting their involvement in RA pathogenesis.

Key words

rheumatoid arthritis, T-cell, GMCSF, polyfunctionality, methotrexate

Aastha Khullar, MSc
Varun Dhir, MD, DM
Biman Saikia, MD
Ashok Kumar Yadav, PhD
Bidyalaxmi Leishangthem, PhD
Chandra Bhushan Prasad, MD, DM
Sankar Jayaprakash, DNB, DM
Siddharth Jain, MD, DM
GSRSNK Naidu, MD, DM
Shefali K. Sharma, MD
Aman Sharma, MD
Sanjay Jain, MD, DM

Please address correspondence to:
Varun Dhir
Division of Rheumatology,
Department of Internal Medicine,
PGIMER,
Chandigarh 160012 India.
E-mail: varundhir@gmail.com

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ORCID iD:
V. Dhir: 0000-0002-3626-0694
A. Khullar: 0000-0002-5002-6124
B. Saikia: 0000-0001-7181-6064
A. Kumar Yadav: 0000-0003-0155-928X
C.B. Prasad: 0000-0003-1594-7873
S. Jain: 0000-0003-4065-5880
GSRSNK Naidu: 0000-0002-9727-044X
S.K. Sharma: 0000-0002-8821-6652
A. Sharma: 0000-0003-0813-1243
S. Jain: 0000-0002-0141-7655

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Introduction

Granulocyte macrophage colony stimulating factor (GMCSF) is a haematopoietic growth factor essential for proliferation, differentiation and activation of myeloid cells (1, 2) . It is known to be produced by various cell types such as monocytes, macrophages, granulocytes, neutrophils, fibroblast, endothelial cells, dendritic cells and even T-lymphocytes and acts on myeloid cells via the GMCSF receptor. GM-CSF promotes inflammation, tissue destruction, and inflammatory cytokine production, and activates and promotes the survival of mature myeloid cells (including macrophages, neutrophils, and dendritic cells) in autoimmune inflammatory diseases driven by T-helper-1 and T-helper-17 pathways (3-5).

There has been an increasing recognition that GMCSF producing T-cells may delineate a subset that is involved in inflammation and pathogenesis of various autoimmune diseases (2, 3). Most of the work has been done in multiple sclerosis (MS) (6), with higher frequencies of GMCSF producing T-cells, especially CD8 T-cells, having been demonstrated in the cerebrospinal fluid in MS and experimental autoimmune encephalomyelitis (EAE), also in MS lesions in the brain. In addition, a reduction has been found in their frequencies on treatment with interferon-β (7-9). Finally, knocking out GMCSF has been shown to confer resistance to EAE (10, 11).

There are reports of arthritis flare after GMCSF administration in rheumatoid arthritis (RA) patients (12), and laboratory studies found high synovial expression of GMCSF and elevated levels in synovial fluid (13, 14). In addition, $GMCSF^{-/-}$ mice were found to be resistant to induction of collagen induced arthritis (CIA), suggesting its important role in RA pathogenesis. Following studies in MS, studies in RA found high frequencies of CD4+GMCSF+T-cells in the synovial fluid (SF), which could generate dendritic cells (DC) from monocytes *in vitro,* suggesting an important pathogenic link (15-18). These cells reduced in frequency after anti-TNF treatment (16, 17, 19).

Polyfunctional T-cells were recognised early on as important mediators of protection against infectious agents such as HIV and tuberculosis and important for vaccine response (20-22). In the past few years, these have been suggested to have an important role in autoimmune diseases like arthritis (PsA) and RA, with some studies finding polyfunctionality precedes onset of disease (23-25). Previous studies have also implicated innate lymphoid cells 2 (ILC2) as the possible source of GMCSF (26).

However, there has been little work on CD8+T-cells producing GMCSF in RA, and polyfunctionality of GMCSF producing T-cells. We wanted to focus on this subset, its frequencies in synovial fluid and circulation, polyfunctionality for other cytokines, phenotype and cytotoxic potential and effect of treatment with methotrexate (MTX).

Materials and methods

This was a single-centre study carried out at a university hospital in North India. The Institutional ethics committee of PGIMER approved the study and all procedures were carried out in accordance with Declaration of Helsinki for research in human subjects. A written and informed consent was taken from all participants.

Study design and participants

This study enrolled RA patients (fulfilling the ACR/EULAR2010 criteria), and age (±5 years) and gender-matched healthy controls (HC) (Supplementary Tables S1 and S2). RA patients between 18 and 60 years of age, with disease duration shorter than 5 years, and not receiving conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs), biological DMARDs or targeted synthetic DMARDs in the last six months, were enrolled after consent. In all patients a blood sample was taken, and if undergoing therapeutic arthrocentesis, synovial fluid was also collected on the same day simultaneously. Some RA patients were recruited for longitudinal follow-up to study the effect of methotrexate. In addition, synovial fluid (SF) of some patients of spondyloarthritis (SpA) undergoing therapeutic arthrocentesis was also collected as disease controls. In all patients, demographic and clinical and

laboratory data like age, disease duration, rheumatoid factor, Anti-citrullinated protein autoantibodies (ACPA) and 28-joint counts were recorded in a predesigned pro forma.

Laboratory procedures

- Synovial fluid and peripheral blood mononuclear cell isolation

Mononuclear cells were isolated from heparinised synovial fluid and venous blood by using density gradient centrifugation using a sterile solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 g/mL (Hisep, Himedia). Briefly, synovial fluid (without dilution) and heparinized venous blood (diluted 1:1 with 1x PBS) was layered on to density gradient and centrifuged (550g x 30 minutes at 25ºC). Upon centrifugation, a buffy layer of SFMCs or PBMCs was carefully aspirated and washed twice with 1X PBS (400g x 5 minutes at 4ºC) and suspended in 1mL complete RPMI 1640 medium.

- Ex-vivo stimulation and immunostaining for intracellular production of GMCSF and other cytokines

Mononuclear cells (1 x 10^6 cells/ml) (SFMCs/PBMCs) were stimulated with phorbol-12-myristate-13-acetate (PMA 50ng/ml) and ionomycin (1µg/ml) for 4 hoursat 37° C in 5% CO_2 in the presence of protein transport inhibitor Brefeldin A (Golgi Plug, BD Biosciences). After washing, cells were surface stained by adding fluorochrome labelled antibodies (CD3,CD8,CCR7 and CD45RA, Biolegend, Suppl. Table S3) and kept at 4ºC for 20 minutes. For intracellular cytokine staining, cells were fixed with para-formaldehyde 4.2% (Cytofix, BD Biosciences) for 5 minutes at room temperature, and then were permeabilised using 500 µl of commercial perm/wash buffer containing FBS and saponin (Perm/Wash Buffer, BD Biosciences) solution for 30 minutes at room temperature. After re-suspension of cells in 50 µl perm buffer, fluorochrome labelled antibodies against intracellular cytokine and cytotoxic products (GM-CSF, TNFα, IL-17, IFN-γ, perforin and Granzyme B, all from Biolegend, CA, USA; were added and kept for 20 minutes at 4ºC. Finally, cells were resuspended in 1X PBS for acquisition by flowcytometry.

- Flowcytometry

Cells were acquired on BD LSRFortessa cytometer (BD Biosciences) and analysis was done using BD FACS Diva 8.0.2 software. A minimum of 10,000 events for CD3+CD8+ cells were acquired. For SFMCs, single T-cells were gated (FSC-A *vs*. FSC-H plot) followed by gating of mononuclear cells and live cells (negative events of Zombie NIR fixable live/dead dye, Biolegend). For PBMCs only mononuclear cell gating was done. Subsequently, a dual parameter contour CD3/CD8 plot was used to gate CD8+ (CD3+CD8+) and CD4+ (CD3+CD8-) populations. Using a dual contour plot (SSc/GMCSF), proportion of CD4 and CD8 T-cells expressing GM-CSF was obtained.

CD8+GMCSF+ and CD4+GMCSF+ were gated and polyfunctionality (production of other cytokines) was assessed by initially opening a dual contour plot of TNF-α and IFN-γ. Further more, in each of the quadrants, IL-17 expression was analysed. Separately, memory/naive status was analysed using a dual contour plot of CCR7 and CD45RA on the gated GMCSF+T-cells. Cytotoxic potential was analysed by using a dual contour plot of perforin/granzyme on the gated cells (Fig. 1A and Suppl. Fig. S1). All gating was based on FMO controls. For analysing polyfunctional GMCSF+T-cells, SPICE 6.1 software was used. The antibodies used in this study are listed in Supplementary Table S3.

- ELISA

The level of GM-CSF and IFN-γ (Suppl. Table S4) in serum and SF (stored at -80°C) was determined using ELISA (RnD high sensitivity ELISA kit).

Statistical analysis

Comparison of frequencies of GMCSF+T-cells, polyfunctional T-cells, memory/naive phenotype between paired samples (SF and PB) of the same individual were analysed using Wilcoxon Signed Ranks test. For frequency of PB GMCSF producing T cells between RA and HC, Mann-Whitney U-test was used. Finally change in frequency on follow-up (paired) was analysed using Wilcoxon Signed Ranks test. Statistical analysis was done using GraphPad Prism8 software. Continuous variables were expressed as median (inter-quartile range) or mean (standard deviation), as appropriate. A p-value <0.05 was considered statistically significant.

Results

We recruited a total of 94 patients of RA and 41 healthy controls (HC) for comparing the frequencies of GMCSF producing T-cells in peripheral blood. Among RA patients, synovial fluid (SF) was available in 32 patients with RA, and synovial fluid mononuclear cells could be isolated in 19 (Suppl. Tables S1 and S2). Thus, paired samples of RA SF with RA blood were available in 19 patients.

GMCSF producing T-cells are enriched in RA synovial fluid

We first compared the frequency of GMCSF producing T-cells (post *ex-vivo* stimulation) in peripheral blood (PB) of patients with RA and HC but did not find any significant difference in either CD4+GMCSF (4.5, 5.2%, p=0.450) or CD8+GMCSF frequencies (3.7, 4.1%, p=0.540) (Fig. 1B) However, there was an enrichment of CD8+ GMCSF+ T-cells (5.8, 3.9%, p=0.0045) and CD4+GMCSF+T-cells in RA-SF (8.5, 4.5%, p=0.0008) compared to RA blood (Fig. 1C). We then compared frequencies of GMCSF producing T-cells in RA-SF with SpA-SF, but found no significant difference in frequencies (numerically higher in RA) (Fig. 1D, Suppl. Fig. S4). The level of cell-free GMCSF was also higher in the RA-SF compared to RA serum (9.5, 3.2 pg /ml, p<0.0001) (Fig. 1E, Suppl. Table S4). Also, cell free GMCSF was elevated in RA-SF compared to SpA-SF. (9.5, 6.55 pg/ml, p=0.009) (Fig. 1E, Suppl. Table S4).

GMCSF producing T-cells in synovial fluid of RA are highly polyfunctional

Next we looked at whether GMCSF producing T-cells were also producers of other inflammatory cytokines, *i.e.* were polyfunctional. We found higher

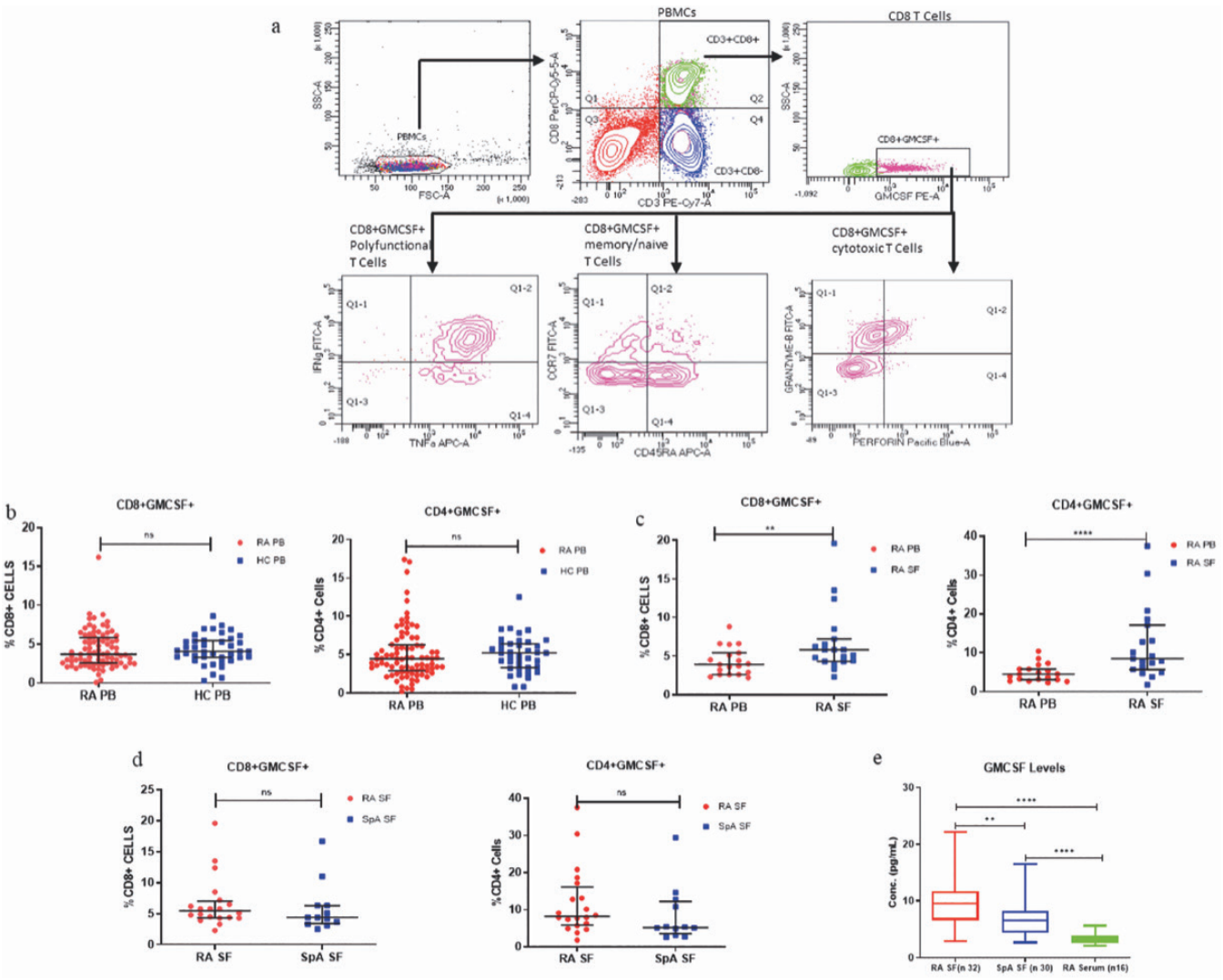

Fig. 1. a. Flowchart of gating strategy for GMCSF producing CD8 T cells, polyfunctional production of other cytokines, memory/naive phenotype based on CCR7/CD45RA and cytotoxic ability (perforin/granzyme) in peripheral blood (CD3+CD8- T cells were taken as CD4+ T cells and gated similarly); **b.** comparison between GMCSF+ T-cells in PB of RA and HC; **c.** comparison between GMCSF+ T-cells in RA patients PB and SF; **d.** comparison between GMCSF+ T-cells in SF of RA and SpA; **e.** GMCSF levels by ELISA in RA SF, SpA SF and RA serum.
RA: rheumatoid arthritis; SpA: spondyloarthritis; SF: synovial fluid; PB: peripheral blood, HC: healthy controls.

frequencies of polyfunctional triple cytokine positive GMCSF+CD8+T-cells (also producingTNF-α+, IFNγ+) (81, 36%, *p*=0.049) and GMCSF+CD4+T-cells (48, 32%, *p*=0.010) in RA-SF compared to RA blood. Additionally, there were significantly higher frequencies of polyfunctional four-cytokine positive CD8+T-cells (also producing TNF-α, IFNγ, IL-17) in RA-SF compared to RA blood(3, 1%, *p*=0.01). (Fig. 2; Suppl. Table S5).

GM-CSF producing T-cells in synovial fluid have effector memory phenotype and display higher cytotoxic potential

Then, we examined the memory phenotype and cytotoxic potential of GMCSF producing T-cells. We found a majority of T-cells producing GMCSF had effector memory (EM) phenotype (CCR7-CD45RA-) in both the SF and peripheral blood, with higher frequency of EM phenotype in RA-SF for both CD8+GMCSF+ (61.5%, 41.8%, *p*=0.0042) and CD4+GMCSF+ T-cells (88.6, 71.9%, *p*=0.0066) (Fig. 3A-B; Suppl. Table S6). Synovial fluid CD8+GMCSF+ T-cells was also found to have a significantly higher expression of granzyme-B than peripheral blood (37%, 17.6%, p=0.0043) but there was no significant difference in perforin expression (Fig. 3C; Suppl. Table S7).

There is a decline in the GMCSF producing T-cells with methotrexate treatment

Finally, to evaluate the effect of methotrexate (MTX), on the frequency of GMCSF producing T-cells, 39 naive RA patients were longitudinally followed up after starting methotrexate treatment. Disease activity score (median, IQR) showed a reduction after MTX treatment DAS28-CRP [5.9 (5.0–6.5), 4.0 (2.8–4.8)] and DAS28-ESR [6.2 (5.8–6.9), 4.7 (3.8-5.6)]. Concurrently with this, there was a significant reduction in GMCSF producing CD4 (4.6% to 2.9%, *p*=0.0014) and CD8 (trend-to) (3.7% to 2.9%, *p*=0.068) T-cells in peripheral blood (post *ex-vivo*

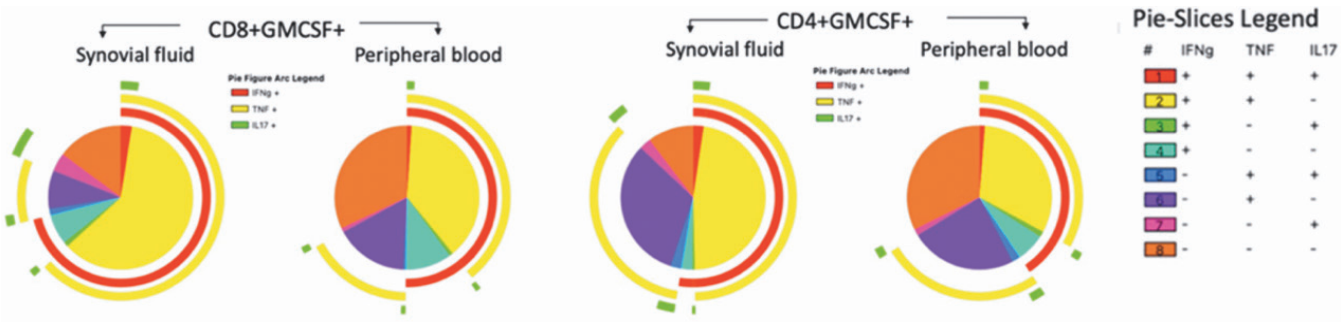

Fig. 2. SPICE diagram showing polyfunctionality of CD8+GMCSF+ and CD4+GMCSF+T-cells. (Pie-slices show the fraction of T-cells expressing combinations of cytokines, whereas pie-arcs show individual cytokine production in T-cells).

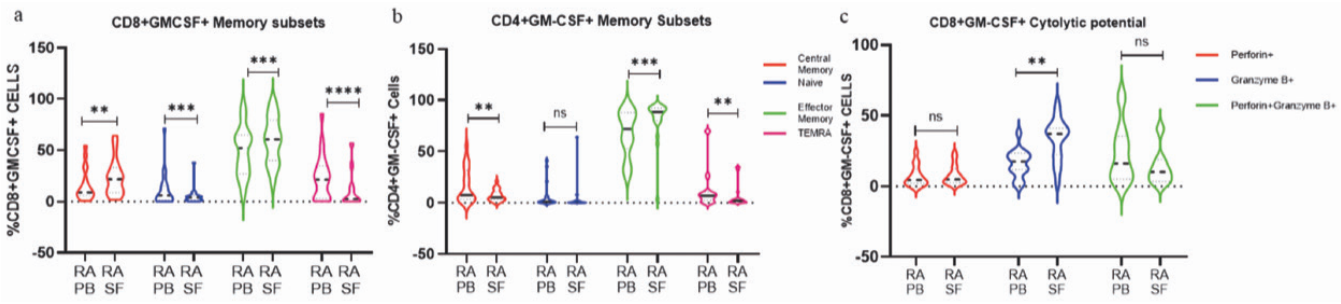

Fig. 3. Violin-plots showing the GMCSF producing T-cells with respect to their memory/naive status in the blood and synovial fluid of RA patients (**a** and **b**), and expression of granzyme and perforin in CD8+GMCSF+ cells (**c**).

stimulation) (Fig. 4). However, there was no direct correlation between these (data not shown).

Discussion

In this study we demonstrate for the first time, higher frequencies of GMCSF producing CD8+ T-cells (post *ex-vivo* stimulation) in rheumatoid SF- with significantly higher cytokine polyfunctionality and cytotoxic potential. A significant reduction after treatment with MTX occurred in frequency of these cells in circulation.

We found significant enrichment of GMCSF-producing CD4 and CD8 T-cells in the SF of RA patients, with approximately 1.5 to 2 times higher frequencies in RA-SF compared to blood. Previously, studies have reported high frequencies of synovial CD4+GMCSF+T-cells, but none have looked at CD8+T-cells (15, 27). Studies have also shown that the CD4+GMCSF+T-cells may have an important role in activation and maturation of DCs (15, 28). It is likely that CD8+GMCSF+T-cells may also enhance the function of antigen presenting cells (APCs) and propagate inflammation, although this needs to be demonstrated. Interestingly, we found a no significant difference in frequency of GMCSF producing T-cells in SpA-SF with RA-SF (although numerically higher in RA), which may suggest a convergent pathophysiological mechanism for both these (29, 30).

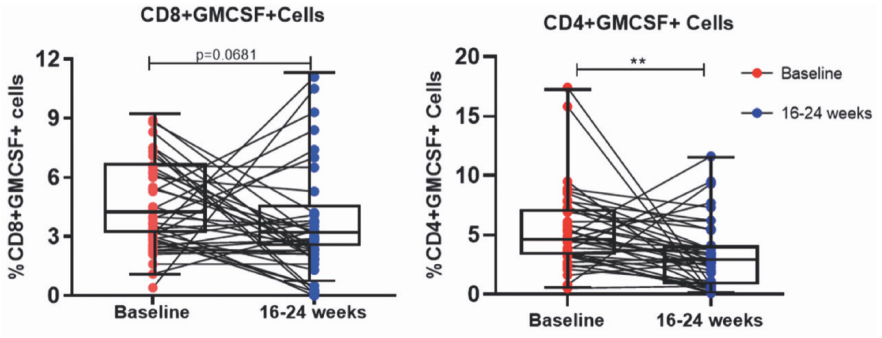

Fig. 4. Before-after plots showing the frequencies of GMCSF producing T-cells at baseline and after methotrexate treatment in the peripheral blood of RA patients (n=39).

We found synovial GMCSF-producing CD8 (and CD4) T-cells to be highly polyfunctional, also producing TNF-α and IFNγ (post *ex-vivo* stimulation). Compared to blood, SF had twice the frequency of polyfunctional cells (around 80% for CD8+GMCSF+ T-cells). High frequency of polyfunctional T-cells has been previously reported in the synovial tissue in PsA, and found to correlate with disease activity (23). However, we did not find any correlation with disease activity in RA. Previously in RA also polyfunctionality of CD4+ T-cells in SF has been demonstrated (for TNF-α, IFNγ, IL21), but none has focussed on GMCSF-producing T-cells, not on CD8 T-cells (24, 27). We found most of the GMCSF producing T-cells belonged to the Tc1 and Th1 subset, whereas only a small minority produced IL-17, which is consistent with recent studies in RA and MS. In contrast some early work especially in animal models suggested that this subset was part of Th17 subset (7, 15, 30). Most of the GMCSF producing T-cells

had an effector memory phenotype, suggesting that they were antigen experienced. CD8+T-cells producing GMCSF had higher expression of granzyme B signifying they were potentially more cytotoxic (16). Finally, this study found a reduction in frequency of GMCSF producing T-cells in the blood of treatment-naïve RA patients (post *ex-vivo* stimulation) after methotrexate (MTX) treatment. Previously it was shown that MTX reduces production of pro-inflammatory cytokines by T-cells, including IFNγ, TNF-α and GMCSF (31). An earlier study also found reduction in frequencies of this subset with combination therapy of TNFi and MTX, but not with MTX alone, explained by their inclusion of MTX non-responders (17, 19) .

This is an exciting time for GMCSF, discovered as a haematopoietic growth factor, but found to have important role in pathogenesis of autoimmune disease. It has opened the door for targeting GMCSF for autoimmune disease control, with biologicals targeting GMCSF (Mavrilimumab, Namilumab, Lenzilumab) (32) some having completed phase II trials, and ongoing phase III trials, these may be licensed in the near future. (33-35). In addition, the currently marketed JAK-inhibitors, although varying in specificity for various JAKs, also target JAK2 which is downstream of the GMCSF receptor, and may owe some part of their action to blocking downstream of GMCSF. The higher level of GMCSF *per se* in the RA-SF is consistent with the effectiveness of these directed therapies.

We hypothesise that at least some of this anti-GMCSF efficacy for RA comes from targeting GMCSF produced by T-cells. It is likely that T-cells on exposure to putative auto-antigen start producing GMCSF, which has an important role in propagating the autoimmune inflammation. It probably works by leading to activation and maturation of antigen presenting cells which augments the aberrant immune response. In addition, the high degree of polyfunctionality for other inflammatory cytokines probably activated multiple inflammatory pathways. Thus, this subset (GMCSF producing T-cells) may be an important future target, and needs further phenotypic characterisation for surface markers.

To conclude, we show here for the first time that CD8 T-cells producing GMCSF apart from CD4+T-cells, were enriched in RA-SF. SF GMCSF producing T-cells demonstrated high polyfunctionality for other inflammatory cytokines, were of the effector memory phenotype and had higher granzyme-B expression. On longitudinal follow-up, methotrexate therapy led to a significant reduction in frequencies of CD4+GMCSF+ T-cells. It is likely that these cells may play an important role in RA pathogenesis.

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What is already known about this subject?

- In the last two decades, GMCSF producing T-cells have been established as important players in pathogenesis of autoimmune disease; initial work in multiple sclerosis found increased frequency of these cells in CSF and plaques.
- In RA, studies have found high frequencies of CD4+T-cell producing GMCSF in synovial fluid (after *ex-vivo* stimulation), which reduced after anti-TNF therapy, but little work on GMCSF+ CD8 T-cells.
- Polyfunctionality may be an important marker of pathogenicity in T-cells

What does this study add?

- Novel finding of increased frequency of GMCSF producing CD8+T-cells (on *ex-vivo* stimulation) in addition to GMCSF+CD4+T-cells in RA synovial fluid compared to RA peripheral blood.
- These cells were highly polyfunctional, with higher frequencies of triple cytokine producing (GMCSF+TNF-α+IFNγ+) in RA synovial fluid, and a majority had effector memory phenotype.
- The frequency of GMCSF+CD4+T-cells in peripheral blood significantly reduced after treatment with methotrexate, and a similar trend in GMCSF+CD8+T-cells.

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