Synovial monocytes from psoriatic and rheumatoid arthritis patients are modulated differently by TNF inhibitors and glucocorticoids: an *ex-vivo* study

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Abstract

Objective

Synovial monocytes (expressing $CD14^+CD16^+$) affect pro-inflammatory responses in the synovium microenvironment of psoriatic arthritis (PsA) and rheumatoid arthritis (RA). The effect of various drugs on those cells was evaluated.

Methods

Synovial fluid mononuclear cells (SFMCs) from PsA (n=29) and RA (n=11) patients were cultured with biologics or glucocorticoids (GCs). $CD14^+CD16^+$ cells were analysed by flow cytometry. TNF secretion was assessed by ELISA and changes in cytokine and matrix metalloproteinase-9 (MMP-9) mRNA by qPCR.

Results

TNF inhibitors (i) [adalimumab (ADA) and infliximab (IFX)] significantly reduced the $\%CD14^+CD16^+$ cells ($p<0.04$ and $p<0.02$, respectively) compared to IL-17Ai, IL-12/23i, and GCs in PsA patients' SFMCs. Similarly, those TNFi reduced the $\%CD14^+CD16^+$ cells ($p<0.05$ and $p<0.02$, respectively) compared to IL-6Ri, CD20i and GCs in RA patients' SFMCs. TNFi (ADA $p<0.01$, IFX $p=0.0003$), and GCs ($p<0.05$) reduced TNF levels in PsA patients SFMCs supernatants. IFX down-regulated IL-1β mRNA ($p<0.005$) while GCs betamethasone (BET) ($p<0.01$) and methylprednisolone acetate (MPA) ($p<0.005$) led to IL-1β up-regulation. IFX down-regulated IL-8 and MMP-9 ($p<0.01$) and up-regulated IL-10 ($p<0.005$), and GCs did so to a greater extent (for IL-8, BET $p<0.0001$ and MPA $p<0.005$, for MMP-9, BET and MPA $p<0.0001$ and for IL-10, BET and MPA $p<0.0001$).

Conclusion

TNFi but not GCs reduced the inflammatory monocytes. Both TNFi and GCs inhibited TNF secretion but differently modulated IL-1β, IL-8, MMP-9 and IL-10 gene expression. Our data point to TNFi as a modulator of synovial monocytes.

Key words

psoriatic arthritis, synovial fluid, monocytes, glucocorticoids, tumour necrosis factor inhibitors

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Introduction

Psoriatic arthritis (PsA) and rheumatoid arthritis (RA) are rheumatic diseases characterised by synovial inflammation. Several cell types promote the synovial inflammation such as: fibroblasts, lymphocytes, neutrophils, and monocytes trafficking into the synovium (1). Three monocyte subsets were classified based upon cell surface marker expression of lipopolysaccharide (LPS)-receptor CD14 and the Fc gamma III receptor CD16: $CD14^+CD16^+$ ("classical"), $CD14^+CD16^+$ ("intermediate"), and $CD14^+CD16^+$ ("non-classical"). In the blood, those subsets distribution is ~85% classical, ~5% intermediate monocytes (IM), and ~10% non-classical (2). In the circulation, IM monocytes are upregulated in PsA and RA, and they correlate with higher disease activity (3, 4).

During inflammation, circulating monocytes migrate to the synovium (5), thereby, monocytes represent 20–40% of the total synovial fluid (SF) cellular content (6, 7). In addition, 70% among synovial monocytes are IM monocytes while the remaining 30% are classical monocytes and the non-classical monocytes are infrequent (6, 8). IM monocytes are the most pro-inflammatory monocyte subset, since they produce the pro-inflammatory cytokines: interleukin 1β (IL-1β), tumour necrosis factor-α (TNF-α), and IL-6 (9). These monocytes are able to differentiate into inflammatory macrophages (M1) which promote T helper 17 (Th17) cells. The latter cells induce RANKL production and initiate osteoclastogenesis (8). IM monocytes express high levels of human leukocyte antigen–antigen D related (HLA-DR), involved in antigen presentation to $CD4^+$ T cells (10).

IM monocytes in SF are more prevalent in RA compared to healthy (11) and are enriched in SF compared to circulation in PsA (12), RA (8) and juvenile idiopathic arthritis (JIA) (13).

Biological therapies have dramatically changed rheumatic diseases management. According to the European League Against Rheumatism (EULAR) guidelines for management of PsA, the following biological agents are included into the treatment repertoire: TNF inhibitors (i), IL-17Ai, and IL-12/23i (14). In RA management they are TNFi, IL-6R (receptor)i, B cell depleting (CD20)i, and selective T cell co-stimulation modulator (15). TNFi therapy was proven to be effective for reducing inflammation and clinical symptoms for both PsA and RA (16). TNFi neutralises inflammatory TNF cytokines (17) but it also exerts activity on monocytes and T cells through binding to transmembrane (tm)TNF in a process known as reverse signaling (18, 19). We had earlier shown TNFi ability to reduce T cell functions of peripheral blood mononuclear cells (PBMCs) derived from PsA patients *in vitro*. TNFi, but not other biologics with different mechanisms of action, reduced T cell activation and proliferation as well as the inflammatory cytokines secretion (20).

Synovial inflammation is commonly reduced by an intra-articular (IA) injection of glucocorticoids (GCs) (21). In this study, we compared different biologics and GCs effect on monocytes derived from synovial fluid mononuclear cells (SFMCs) of PsA and RA patients. Those SFMCs were co-cultured *ex-vivo* with the experimental drugs at the relevant therapeutic concentrations, and changes in $\%CD14^+CD16^+$ and $\%CD14^+HLA\text{-}DR^+$ cells were determined. In addition, TNF cytokine levels in supernatants were analysed, as well as the effects of the drugs on modulation of pro- and anti-inflammatory cytokine gene expression.

Material and methods

Study population and ethics

The study population included PsA and RA patients. SFMCs were obtained from synovial fluid derived from PsA and RA patients with at least one swollen joint. The arthrocentesis procedure was performed by an expert rheumatologist at the Department of Rheumatology, Tel Aviv Sourasky Medical Center (TASMC). All PsA patients were diagnosed by the classification for Psoriatic Arthritis (CASPAR) study group criteria (22). The RA patients were diagnosed based on the criteria of the American College of Rheumatology for RA (23). The patient's demographic and clinical parameters

Table I. Demographic and clinical features of PsA and RA patients.

	PsA (n=29)	RA (n=11)
Sex, F/M	7/22	8/3
Age, mean ± SEM (years)	49.6 ± 2.5	52.4 ± 6.2
White blood cell count (cells/µl) in the synovial fluid sample, mean ± SEM	31520 ± 5150	25500 ± 5700
SJC	2.5 ± 0.6	3.0 ± 0.7
TJC	2.7 ± 0.6	3.4 ± 1.0
Treatment	Untreated = 10 Methotrexate = 5 Infliximab = 2 Adalimumab = 4 Etanercept = 1 Secukinumab = 2 Ustekinumab = 1 Apremilast = 1 Tofacitinib = 2 Upadacitinib = 1	Untreated = 4 Methotrexate = 2 Plaquenil + Prednisone = 1 Leflunomide = 1 Etanercept = 2 Rituximab = 1 Tocilizumab = 1

F: female; M: male; SEM: standard error of the mean; SJC: swollen joint count; TJC: tender joint count.

are summarised in Table I. This study was approved by the TASMC ethics board (0182-18-TLV) and conducted according to the principles of the Declaration of Helsinki. Written informed consent was signed by all participants.

Isolation and culture of SFMCs ex-vivo

PsA and RA patients SFMCs were isolated by Lymphoprep (Axis-Shield, Oslo, Norway) density-gradient centrifugation. SFMCs in the interphase were collected and resuspended at 1.5–2 x10^6 cells/ml in RPMI 1640 medium (Biological industries, Kibbutz Beit Haemek, Israel) with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO_2. SFMCs derived from PsA patients were cultured with the following biologics: adalimumab (ADA) (Humira®, Abbvie, Chicago, USA), infliximab (IFX) (Remicade®, Janssen Biologics, Leiden, The Netherlands), secukinumab (SEC) (Cosentyx®, Novartis, New Jersey, USA), or ustekinumab (UST) (Stelara®, Janssen, Schaffhausen, Switzerland) at 10 µg/ml or with the GCs: betamethasone (BET) (Diprospan®, Schering-Plough, Heist-op-den-berg, Belgium) and methylprednisolone acetate (MPA) (Depo-Medrol®, Pfizer, New-York USA) at 1 µg/ml or 10 µg/ml. SFMCs derived from RA patients were cultured with the same agents but tocilizumab (TCZ) (Actemra®, Roche, Mannheim, Germany) and rituximab (RTX) (MabThera®, Roche) replaced the SEC and UST. Cells were cultured for 48 hours for mRNA expression analysis, 4 days for changes in %$CD14^+HLA\text{-}DR^+$, and 7 days for $CD14^+CD16^+$-expressing cells as determined by flow cytometry analysis. Supernatants were collected after 7 days in culture and analysed for TNF secretion by ELISA.

Flow cytometry

SFMCs were stained with the following antibodies: FITC-anti-human CD14 (BioLegend, San Diego, CA, USA, cat no. BLG-325604), APC-anti-human CD16 (BioLegend cat no. BLG-302012), or PE-anti-human HLA-DR (BioLegend cat no. BLG-307605) for 30 min at room temperature. Flow cytometry was performed with a FACS Canto™ II instrument (BD Biosciences) and the data were analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Real-time PCR

SFMCs were cultured for 48 hours with the above-mentioned drugs for gene expression analysis. After incubation, cells were collected and total RNA was isolated by an RNA extraction kit (High Pure RNA Isolation Kit, Roche, Mannheim, Germany). For cDNA synthesis, 300 ng total RNA was transcribed with cDNA with the High Capacity cDNA Reverse Transcription Kit (Invitrogen™ , Carlsbad, CA, USA). Gene expression was performed with Fast SYBR™ Green Master Mix (Applied Biosystems™ , CA, USA) in real-time PCR with StepOnePlus™ Real-Time PCR System (Applied Biosystems). All procedures were performed according to the manufacturer's instructions. The following human primers were used: (forward and reverse, respectively): IL-1β 5′-TGATGGCTTATTACAGTGGCAATG-3′ and 5′-GTAGTGGTGGTGGGAGATTCG-3′, IL-8 5′-ACTGAGAGTGATTGAGAGTGGAC-3′ and 5′-AACCCTCTGCACCCAGTTTTC-3′, IL-10 5′-TGGAGGACTTTAAGGGTTAC-3′ and 5′-GATGTCTGGGTCTTGGTT-3′, MMP-9 5′-TTGACAGCGACAAGAAGTGG -3′ and 5′-GCCATTCACGTCGTCCTTAT-3′ and GAPDH 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-GGGGTCATTGATGGCAACAATA-3′. The GAPDH levels were used to normalise gene expression levels.

ELISA

Supernatants from *ex-vivo* 7 days SFMCs co-culture with the experimental drugs were harvested, underwent centrifugation at 450 g for 5 min and supernatants were stored at -80°C for further analysis. TNF levels were measured with ELISA DuoSet kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SE. Non-parametric analyses were performed with the Mann-Whitney U-test or Kruskal-Wallis followed by Dunn's multiple comparison test. Statistical significance was determined when the *p*-value was <0.05, and all analyses were performed with GraphPad Prism software v. 8 (San Diego, CA, USA).

Results

Comparison between different biologics used in PsA management and GCs on inflammatory monocytes derived from SFMCs of PsA patients

SFMCs derived from PsA patients (n=22) were co-cultured *ex-vivo* with

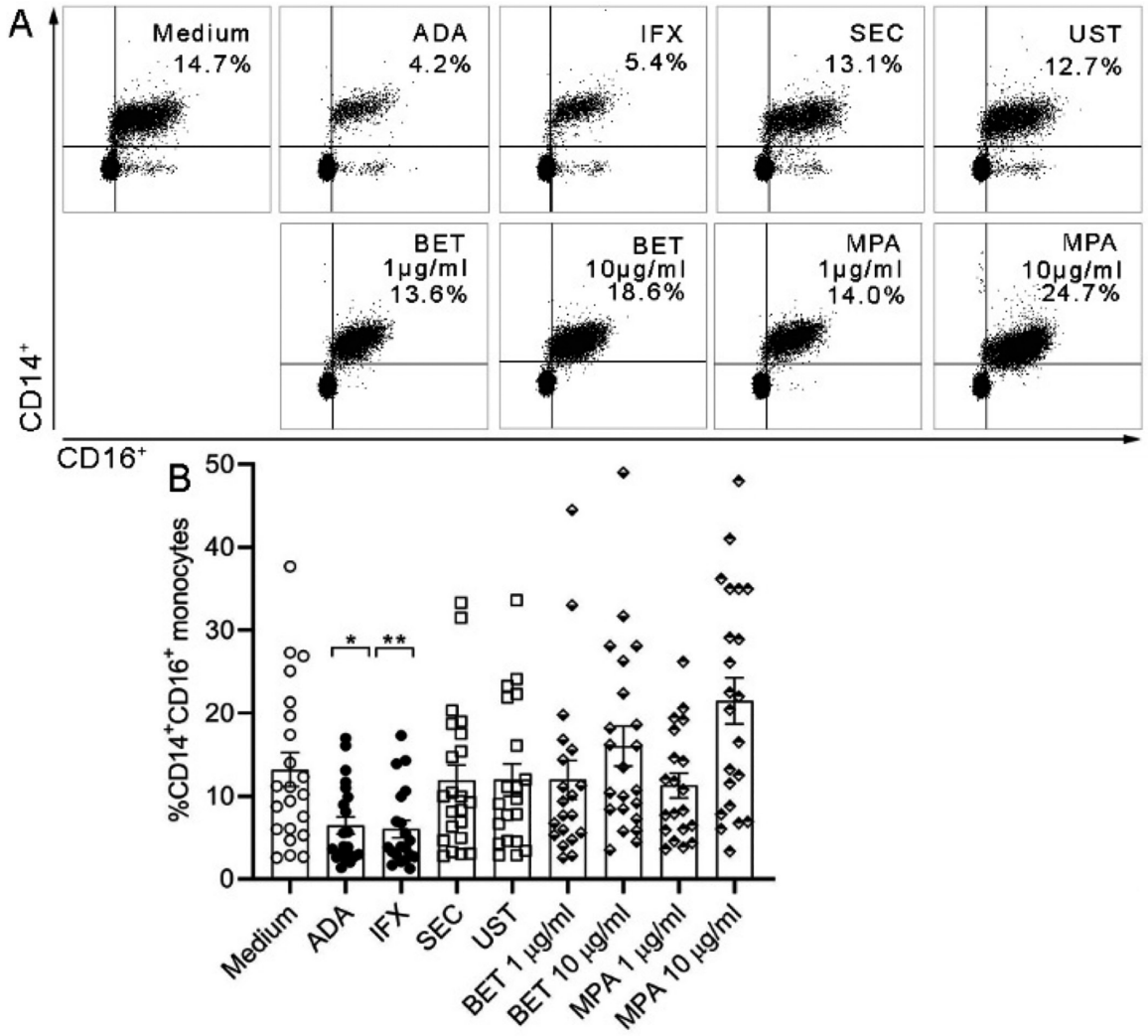

Fig. 1. %CD14$^+$CD16$^+$ monocytes derived from PsA patients SFMCs are reduced by TNFi *ex-vivo*. SFMCs derived from PsA patients (n=22) were co-cultured *ex-vivo* for 7 days with adalimumab (ADA), infliximab (IFX), secukinumab (SEC), and ustekinumab (UST) at 10 µg/ml or with betamethasone (BET) and methylprednisolone (MPA) at 1 µg/ml and 10 µg/ml or with medium alone.
A: Representative flow cytometry plots of PsA patient-derived SFMCs labelled with anti-CD14 and anti-CD16 after 7 days in culture with medium alone or with therapeutic agents as indicated. Values in the upper-right quadrant of each plot represent the %CD14$^+$ CD16$^+$ cells.
B: Graphic representation of %CD14$^+$CD16$^+$ monocytes. Comparisons between the indicated groups are shown. Mean ± SEM are indicated. Significance was assessed with the non-parametric one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test, *p<0.04, **p<0.02.

either TNFi [(adalimumab, ADA), (infliximab, IFX), IL-17Ai (secukinumab, SEC), or IL-12/23i (ustekinumab, UST) (10 µg/ml)] or with GCs [betamethasone (BET) and methylprednisolone acetate (MPA) both at 1 µg/ml and 10 µg/ml)]. A sample with medium alone, *i.e.* without any drug, was used as a control. Cells were incubated for 7 days and then analysed by flow cytometry to determine the %CD14$^+$CD16$^+$ cells. Figure 1 demonstrates that ADA or IFX supplementation to SFMCs culture significantly reduced the %CD14$^+$CD16$^+$ cells (6.4±1.0, p<0.04 and 6.0±1.0, p<0.02, respectively) compared to the medium alone (13.2±2.0), representing a 50% decrease. In contrast, SEC or UST (11.9±1.8 and 12.0±2.0, respectively) did not affect the %CD14$^+$CD16$^+$ cells as compared to the medium. In addition, both GCs at both concentrations did not change the %CD14$^+$CD16$^+$ cells compared to the medium (BET 1 µg/ml 12.0±2.3, BET 10 µg/ml 16.0±2.4, MPA 1 µg/ml 11.3±1.5 and MPA 10 µg/ml 21.5±2.8). More-over, GCs at a concentration of 10 µg/ml non-significantly increased the %CD14$^+$CD16$^+$ cells compared to the medium.

The effect of different therapeutic agents on the %CD14$^+$HLA-DR$^+$ cells in PsA patients derived SFMCs (n=7) after 4 days in culture was also evaluated. IFX supplementation to SFMCs culture reduced the %CD14$^+$HLA-DR$^+$ cells (4.7±0.6) (p=0.01), whereas SEC, BET, or MPA (1 µg/ml) did not affect the %CD14$^+$HLA-DR$^+$ cells (6.8±0.6, 9.1±2.7 and 8.8±2.2, respectively) compared to the medium (7.0±0.6) (Fig. 2).

Comparison between different biologics used in RA management and GCs on inflammatory monocytes derived from SFMCs of RA patients

SFMCs derived from RA patients (n=11) were co-cultured *ex-vivo* with both TNFi (ADA) and (IFX), IL-6Ri (TCZ), and CD20i (RTX) (10 µg/ml)

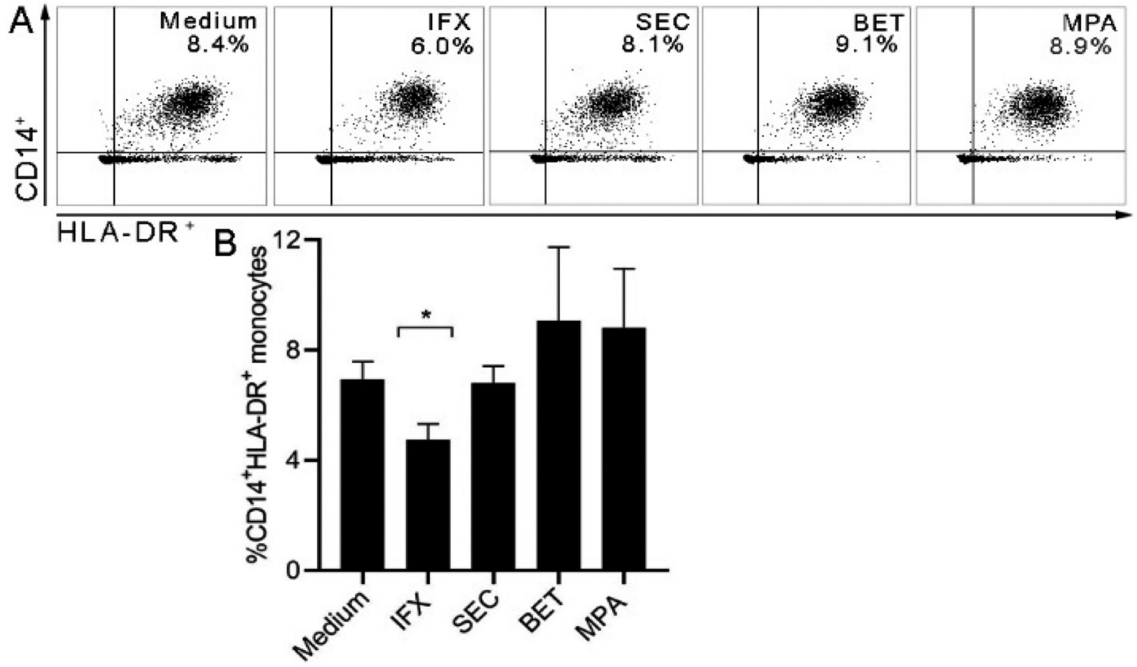

Fig. 2. %CD14⁺HLA-DR⁺ monocytes derived from PsA patients SFMCs are reduced by TNFi *ex-vivo*. Effect of infliximab (IFX), secukinumab (SEC) at 10 μg/ml or with betamethasone (BET) and methylprednisolone (MPA) at 1 μg/ml on %CD14⁺HLA-DR⁺ cells in PsA patient-derived (n=7) SFMCs after 4 days in culture.
A: Representative flow cytometry plots of PsA patient-derived SFMCs labelled with anti-CD14 and anti-HLA-DR mABs after 4 days in culture with medium alone or with the therapeutic agents as indicated. Values in the upper-right quadrant of each plot represent the %CD14⁺ CD16⁺ cells.
B: Bars represent the mean ± SEM, the Mann-Whitney U-test was used in order to analayse difference among groups, **p*=0.01.

or with BET and MPA at 1 μg/ml and 10 μg/ml. A sample with medium alone was used as a control. A similar trend was observed after supplementation of ADA or IFX to SFMCs culture derived from RA patients (Fig. 3). Both ADA and IFX significantly reduced the %CD14⁺CD16⁺ cells (5.1±0.8, $p<0.05$ and 4.8±0.6, $p<0.02$, respectively) compared to the medium alone (8.2±0.8). The biologic agents, TCZ or RTX did not affect the %CD14⁺CD16⁺ cells (8.9±1.0 and 8.4±0.8, respectively) as compared to the medium alone. Moreover, as shown in Figure 3, GCs (BET 10 μg/ml, 22.1±3.5, $p<0.02$ and MPA 10 μg/ml, 27.8±4.1, $p<0.002$) significantly increased the %CD14⁺CD16⁺ monocytes as compared to the medium alone, whereas GCs at the lower concentration (1μg/ml) increased the %CD14⁺CD16⁺ monocytes but non-significantly (BET 1 μg/ml, 17.4±3.6 and MPA 1 μg/ml 19.8±3.8).

Both TNFi and GCs inhibit TNF cytokine in SFMCs supernatants derived from PsA patients ex-vivo

The 4 different biologics used for PsA therapy (ADA, IFX, SEC and UST at 10 μg/ml) were co-cultured with SFMCs derived from PsA patients to test their effects and compare them to GCs (BET and MPA at 1 μg/ml and 10 μg/ml) on TNF cytokine production. The TNF level was significantly decreased by both the TNFi, ADA and IFX (18.1±1.4, $p<0.01$ and 15.3±0.8, $p<0.0003$, respectively) as compared to the medium alone (93.7±12.8) (Fig. 4). GCs were also able to inhibit TNF cytokine production in those cultures as compared to the medium alone (BET 1 μg/ml 22.2±4.0, $p<0.01$, BET 10 μg/ml 24.0±4.2, $p<0.04$, MPA 1 μg/ml 28.3±4.2, $p<0.01$, and MPA 10 μg/ml 31.2±10.1, $p<0.01$). However, the two other biologics, IL-17Ai (SEC) and IL-12/23i (UST), did not affect the level of TNF as compared to the medium alone (117.8±21.0 and 82.9±14.3, respectively).

TNFi and GCs modulate differently gene expression in SFMCs derived from PsA patients ex-vivo

The potential of TNFi to inhibit the %CD14⁺CD16⁺ monocytes compared to GCs in SFMCs culture (demonstrated by the first set of experiments) led us to investigate the drugs mode of action in modulating pro-inflammatory (IL-1β, IL-8 and MMP-9) and anti-inflammatory (IL-10) gene expression. For this purpose, we examined the potency of TNFi (IFX), IL-17Ai (SEC), BET, and MPA (1 μg/ml) to modulate gene expression following 48 hours in co-culture with SFMCs derived from PsA patients. The relative expression of inflammatory IL-1β was significantly reduced by IFX (0.51±0.03, $p<0.01$) as compared to the medium alone (1.0±0.02). In contrast, both GCs significantly increased IL-1β expression as compared to the medium alone (BET and MPA 1.5±0.07, $p<0.01$ and 1.7±0.14, $p<0.005$, respectively) (Fig. 5). IL-17Ai (SEC) did not modulate IL-1β expression as compared to the medium (1.0±0.1) (Fig. 5A). The inflammatory IL-8 and MMP-9 were significantly reduced by both GCs (for IL-8: BET 0.4±0.4, $p<0.0001$ and MPA and 0.6±0.1, $p<0.005$, for MMP-9: BET 0.1±0.02, $p<0.0001$ and MPA 0.18±0.02, $p<0.0001$) as well as by TNFi (for IL-8: 0.7±0.1, $p<0.01$, for MMP-9: 0.4±0.04, $p<0.01$) as compared to the medium alone. As shown

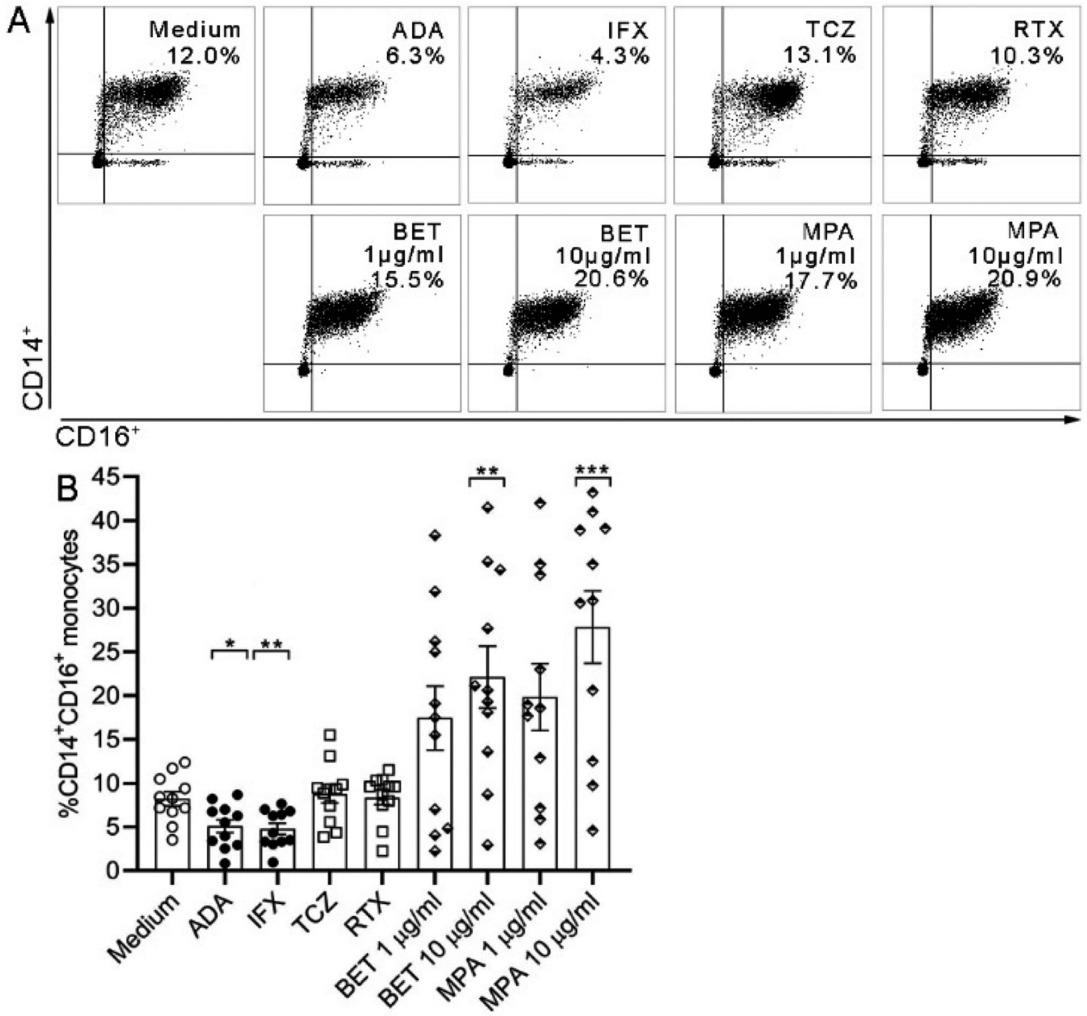

Fig. 3. %CD14+CD16+ monocytes derived from RA patients SFMCs are reduced by TNFi *ex-vivo*. SFMCs from RA patients (n=11) were co-cultured *ex-vivo* for 7 days with adalimumab (ADA), infliximab (IFX), tocilizumab (TCZ), or rituximab (RTX) at 10 µg/ml or with betamethasone (BET) and methylprednisolone (MPA) at 1 µg/ml and 10 µg/ml or with medium alone.
A: Representative flow cytometry plots of RA patient-derived SFMCs stained for CD14 and CD16 after 7 days in culture with medium alone or with the therapeutic agents as indicated. Positive staining is presented in the right upper quadrant of each plot with the percentage indicated.
B: Graphic representation of %$CD14^+CD16^+$ monocytes. Comparisons between the indicated groups are shown. Mean ± SEM are indicated. Significance was assessed with the non-parametric one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test, *p<0.05, **p<0.02, ***p<0.002.

in Figure 5, GCs reduced the IL-8 and MMP-9 expression to a greater extent. Consistently with the results of IL-1β, IL-17Ai did not modulate the IL-8 and MMP-9 expression as compared to the medium alone (1.0±0.1), (Fig. 5B and C, respectively). In contrast, the anti-inflammatory IL-10 was significantly elevated by both GCs (BET and MPA 2.7±0.1, p<0.0001 and 3.2±0.1, p<0.0001, respectively) as well as by TNFi (1.7±0.2, p<0.01) (Fig. 5D), although GCs elevated IL-10 expression to a greater extent as compared to the medium alone (1.0±0.1). IL-17Ai did not modulate the IL-10 expression as compared to the medium alone (1.0±0.1).

Discussion

This study demonstrates that TNFi reduces the %$CD14^+CD16^+$ inflammatory monocytes in SFMCs derived from PsA and RA patients. Moreover, the activity of TNFi was selective and not mediated by the tested therapeutic agents, such as GCs, nor by other biologics used in PsA management (IL-17Ai or IL-12/23i) or RA management (IL-6Ri or CD20i). Both TNFi and GCs similarly inhibited the TNF cytokine in SFMCs culture supernatants. Analysis of gene expression revealed a differential mechanism of action for TNFi compared to GCs. IL-1β was down-modulated by TNFi and up-regulated by GCs, whereas inflammatory IL-8 and MMP-9, as well as, the protective IL-10 were modulated in a similar fashion by TNFi and GCs, however GCs modulated them to a greater extent.

TNFi neutralise both soluble and tmTNF which is believed to be related to their therapeutic effect, although an alternative mode of action of TNFi may exist TNFi binds to tmTNF on monocytes and T cells in a process known as reverse signaling (RS) (19, 24). The latter contribute to certain TNFi effects exerted on immune cells, such as migratory inhibition (25) and increased apoptosis (26). RS through tmTNF inhibit the constitutive NF-κB activation in RA monocytes, leading to suppression of IL-1β secretion (27). Indeed,

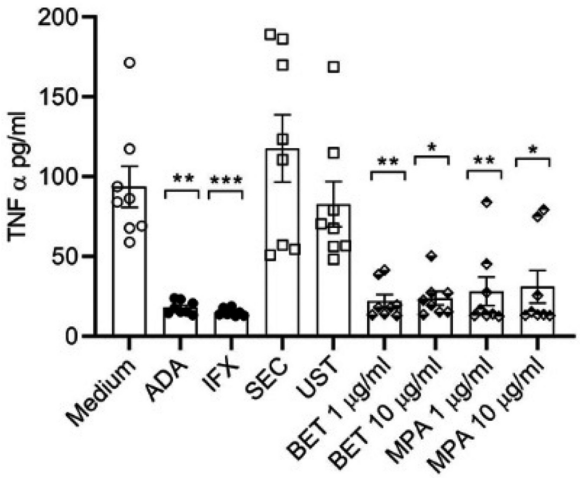

Fig. 4. Both TNFi and GCs block TNF secretion in PsA patients SFMCs *ex-vivo*. SFMCs were co-cultured with adalimumab (ADA), infliximab (IFX), secukinumab (SEC), and ustekinumab (UST) at 10 µg/ml or with betamethasone (BET) and methylprednisolone (MPA) at 1 µg/ml and 10 µg/ml or with medium alone. Supernatants were analysed by enzyme-linked immunosorbent assay (ELISA) for TNF levels (n=8). Results are expressed as mean ± SEM and the statistical analysis was performed with Kruskal-Wallis test followed by Dunn's *post-hoc* comparisons, **p*<0.04, ***p*<0.01, ****p*=0.0003.

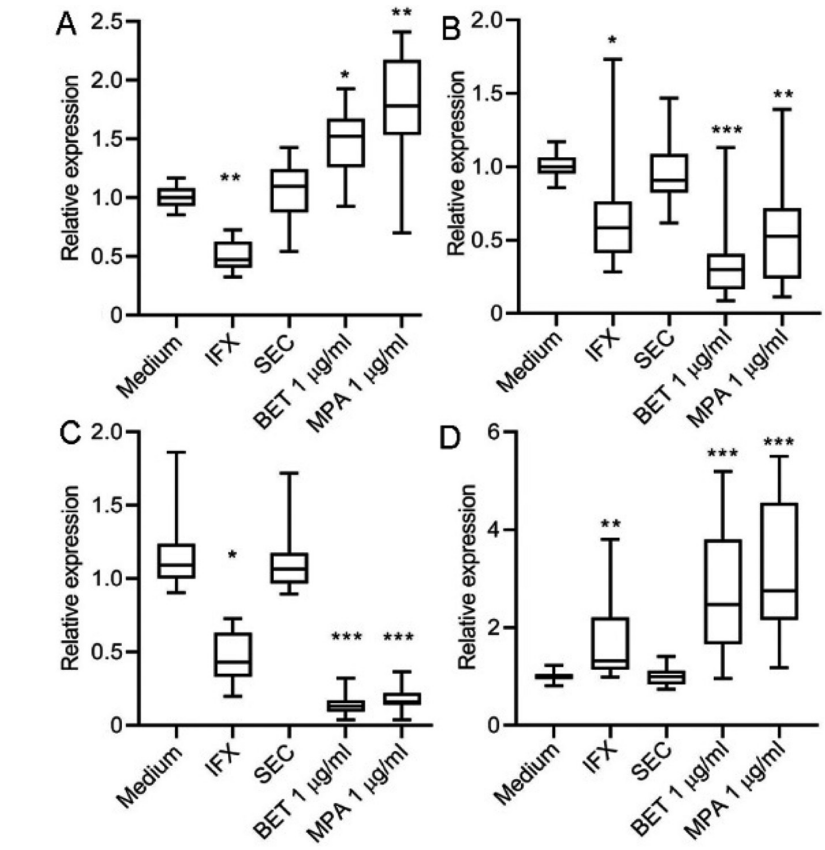

Fig. 5. TNFi modulate cytokines gene expression in PsA patients' SFMCs in a differ mechanism from that of GCs. SFMCs derived from PsA patients (n=6) were cultured ($1.5x10^6$ cells/well) for 48 hours in the presence of TNFi (IFX), IL-17Ai (SEC) at 10 µg/ml, or GCs (BET and MPA) at 1 µg/ml. IL-1β (**A**), IL-8 (B), MMP-9 (**C**) and IL-10 (**D**) mRNA expression was determined by real-time PCR. Data are shown as relative expression, normalised to GAPDH, mean ± SEM, analysed with Kruskal-Wallis test followed by Dunn's *post-hoc* comparisons, **p* 0.01, ***p*<0.005, ****p*<0.0001.

we were able to show that TNFi inhibited IL-1β gene expression in PsA patients' SFMCs *ex-vivo*, and this activity was mediated only by TNFi and not by other drugs tested (*i.e.* GCs or IL-17Ai). IL-1β is overexpressed in arthritic joints (28), this implies that a drug that could block IL-1β like anakinra, an IL-1 receptor antagonist, could attenuate inflammation induced by IL-1β. However, anakinra was also assessed in our *ex-vivo* experiments and it could not reduce the %$CD14^+CD16^+$ monocytes in SFMCs derived from PsA and RA patients compared to the medium control (data not shown).

The significance of tmTNF RS was recently demonstrated in a triple transgenic mouse model (3TG) lacking TNF receptor R1 and R2 expression (TNFR1/R2 KO). Those mice expressed TNF at a physiological level exclusively in tmTNF form due to knock-in mutations. Following arthritis induction, the 3TG mice were treated with TNFi injections and this treatment led to arthritis disease attenuation. Furthermore, pro-inflammatory IL-1β was inhibited, and neutrophils were less numerous in the treated mice joints, thus demonstrating for the first time the RS significance *in vivo* and implicating a novel interpretation for TNFi therapy effect in inflammatory diseases treatment (29).

GCs are widely used for suppression of both acute and chronic inflammation. Their clinical efficacy is mediated by their effect on T cells (30) or monocytes/macrophages (31). They penetrate the plasma membrane due to their lipophilic structure and bind to the cytosolic GC-receptor (GR) which is localised in the cytoplasm in a multi-protein chaperone complex (32). The GC/GR complex is then transported to the nucleus where it functions as transcription factors regulators (33). They inhibit pro-inflammatory cytokines production in monocytes and macrophages as the result of an interaction between GC receptor monomers and pro-inflammatory transcription factors, such as NF-κB or AP-1, in a process called "transrepression" (34). GCs also induce anti-inflammatory mediators in monocytes/macrophages (*e.g.* annexin A1) (35). Importantly, we found that GCs were unable to reduce the %$CD14^+CD16^+$ monocytes in SFMCs *ex-vivo*.

Similarly, others had shown that $CD14^+CD16^+$ IM monocytes in blood of uveitis patients following GCs therapy were enriched but expressed less TNF and more IL-10, and induced IM monocytes expansion *ex-vivo*. The IM monocytes were restricted in their ability to induce T cell activation and proliferation, but rather increased T regulatory cell IL-10 expression and attenuated naive $CD4^+$ T cells proliferation *ex-vivo* (36). GCs also induce an anti-inflammatory phenotype in monocytes from healthy donors *ex-*

vivo (37). Those results are in line with our current ones by showing elevated IL-10 gene expression following *ex-vivo* SFMCs culture with GCs. Mozo *et al*. observed that GCs induce IL-10 secretion from monocytes but not from T cells (38). A study performed on co-culture of RA SF-derived T cells and monocytes, found that the monocytes render the cultured T cells to be resistant to GCs-induced apoptosis. The authors in this study proposed to use combination of locally administrated GCs with monocyte-targeted therapies rather than T-cell targeted therapies for effective attenuation of synovial inflammation (39). Also, when peripheral blood monocytes from multiple sclerosis (MS) patients were cultured with MPA *ex-vivo* (10^{-6}M) for 3 h or isolated from the same MS patients again 24 h after MPA therapy, they had only a minor impact on monocyte subset distribution (40), indicating that GCs do not reduce monocytes. However, the beneficial effect of local GCs treatment in reducing synovial inflammation could derive from their selective activity on two different monocytes/macrophages populations: one is granulocyte macrophage-colony stimulating factor (GM-CSF) monocytes/macrophages that are pro-inflammatory and the other is macrophage-lineage-specific growth factor (M-CSF) monocytes/macrophages that are anti-inflammatory (41). GCs induce apoptosis in GM-CSF monocytes through ERK1/2 signalling pathway induction, while they have no impact on M-CSF-induced monocyte survival, therefore favoring the remaining anti-inflammatory monocytes (42).

The GCs therapeutic benefit in IA injections is well established and Carubbi et al. attempted to assess TNFi utility for AI administration. It emerged that TNFi injections were more effective than GCs in reducing synovial inflammation, as demonstrated by ultrasonic scans, and that they achieved good longstanding clinical and radiological responses as well as disease remission (43). Also, etanercept was superior to saline in IA injections to joints of PsA and RA patients (44). Moreover, a systematic literature review conducted to identify randomised controlled trials assessing IA therapies in RA patients found that TNFi had efficacy equal to that of GCs in IA injections for persistent monoarthritis in RA (45).

This study has a number of limitations. First, IM monocytes were evaluated without assessing other relevant immune cell populations such as modulation of M1/M2 macrophages. Second, patients in this cohort were concomitantly treated with synthetic or biologic DMARDs that could have influenced on the SFMCs immune populations distribution at a degree that cannot be assessed.

In conclusion, there is an increase monocyte representation in synovial cellularity of various arthropathies. TNFi specifically block the $CD14^+CD16^+$ monocytes in SF of patients with different arthropathies. $CD14^+CD16^+$ monocytes are involved in inflammatory cytokine secretion and other inflammatory cells recruitment to the joint. Our data support the utility of TNFi as potential drugs that can assist in re-programming the cellular content in the inflamed synovium and to restore homeostasis. However, more research is needed to investigate their *in vivo* efficacy, including testing the optimal dose and IA TNFi administration frequency, as well as to investigate the TNFi long-term outcome in IA therapy.

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