Human CCR6+ Memory T-helpers Cells form a Heterogenous Population Including Th17/Th22 and Th17.1 Cells, which Differ in Transcription Factor and Cytokine Expression but all Activate Synovial Fibroblast in an IFNγ-independent Manner

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Research Article

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Abstract

Background

Chronic synovial inflammation is an important hallmark of inflammatory arthritis, but the cells and mechanisms involved are incompletely understood. Previously, we have shown that CCR6+ memory T-helper (memTh) cells and synovial fibroblasts (SF) activate each other in a pro-inflammatory feedforward loop, which potentially drives persistent synovial inflammation in inflammatory arthritis. However, the CCR6+ memTh cells are a heterogeneous population, containing Th17/Th22 and Th17.1 cells. Currently it is unclear which of these subpopulations drive SF activation and how they should be targeted. In this study, we examined the individual contribution of these CCR6+ memTh subpopulations to SF activation and examined ways to regulate their function.

Methods

Th17/Th22 ($CXCR3^-CCR4^+$), Th17.1 ($CXCR3^+CCR4^-$), DP ($CXCR3^+CCR4^+$) and DN ($CXCR3^-CCR4^-$) CCR6+ memTh, cells sorted from PBMC of healthy donors or treatment-naïve early rheumatoid arthritis (RA) patients, were cocultured with SF from RA patients with or without anti-IL17A, anti-IFNγ or $1,25(OH)_2D_3$. Cultures were analyzed by RT-PCR, ELISA or flow cytometry.

Results

Th17/Th22, Th17.1, DP and DN cells equally express RORC, but differ in production of T-bet and cytokines like IL-17A and IFNγ. Despite these differences, all the individual CCR6+ memTh subpopulations, both from healthy individuals and RA patients, were more potent in activating SF than the classical Th1 cells. SF activation was partially inhibited by blocking IL-17A, but not by inhibiting IFNγ or T-bet. However, active vitamin D inhibited the pathogenicity of all subpopulations leading to suppression of SF activation.

Conclusions

Human CCR6+ memTh cells contain several subpopulations that equally express RORC but differ in T-bet, IFNγ and IL-17A expression. All individual Th17 subpopulations are more potent in activating SF than classical Th1 cells in an IFNγ-independent manner. Furthermore, our data suggest that IL-17A is not dominant in this T cell-SF activation loop but that a multiple T cell cytokine inhibitor, such as $1,25(OH)_2D_3$, is able to suppress CCR6+ memTh subpopulation-driven SF activation.

Background

Persistent synovial inflammation is a hallmark of inflammatory arthritis such as psoriatic arthritis (PsA) and rheumatoid arthritis (RA). This results in pain, fatigue and functional disability, which cannot be sufficiently suppressed in a large group of patients due to ineffective treatment response or treatment

resistance (1–3). In order to further improve the quality of life of these patients, it is important to understand the underlying pathogenesis and which cells play a role in driving the chronic inflammation.

The relevance of T cell biology in inflammatory arthritis is supported by their infiltration into arthritic joints, and also by various RA associated genes, including MHC class II, PTPN22, CTLA-4 and CCR6, that are related to T cell biology (4–6). Both Th1 and Th17 cells are present in inflammatory arthritis, although which of these cell types drives chronicity of joint inflammation is not fully elucidated (7). Moreover, with the discovery of the newly identified Th17.1 cells, which express T-bet and produce IFNγ but are also RORC positive, the potential role for IFNγ-producing Th17 cells in arthritis needs to be reconsidered (8, 9).

Importantly, both Th17 and Th17.1 cells express the chemokine receptor CCR6. Previously, we and others have shown that CCR6 + memory T-helper (memTh) cells are elevated in the blood of treatment-naïve early RA patients and are present in the synovial fluid (10, 11). These cells express the transcription factor RORC and pro-inflammatory cytokines such as interleukin (IL)-17A, tumor necrosis factor alpha (TNFα) and interferon-gamma (IFNγ) (12). Functionally, they contribute to synovial inflammation by activating synovial fibroblasts (SF), which in turn further activate the CCR6 + memTh cells to create a pro-inflammatory loop. This loop also leads to the induction of IL-8 to attract more immune cells, of prostaglandin-E2 (PGE2), IL-6 and IL-1β to activate other immune cells and of matrix metalloproteases (MMPs) that contribute to the tissue damage (10).

However, CCR6 + memTh cells are a heterogeneous group of cells which can be further subdivided based on their expression of the chemokine receptors CCR4 and CXCR3 (12). Th17/Th22 cells, distinguished as CCR4 + CXCR3- CCR6 + memTh cells, express high levels of IL-17A and (very) low to absent IFNγ, and are RORC and IL-22 positive. Double-positive (DP) cells, expressing both CCR4 and CXCR3, produce both IL-17A and IFNγ and are RORC and T-bet positive. Th17.1 cells, or also called non-classical Th1 cells, are identified as CCR4- CXCR3 + CCR6 + memTh cells and co-express RORC and T-bet, but also high levels of IFNγ and low amounts of IL-17A. Th17.1 cells are specifically elevated at the inflammatory sites in autoimmune diseases such as sarcoidosis, Crohn's disease and juvenile idiopathic arthritis (9, 13, 14). Interestingly, there is also a subpopulation within CCR6 + memTh cells that is less well described. They express neither CCR4 nor CXCR3 receptors (double-negative cells, DN) (12).

Although the CCR6 + memTh are found at inflammatory sites, it is unclear which of the subpopulations is most capable of initiating synovial inflammation. Classically, Th17 cells were thought to be the main drivers of chronic joint inflammation leading to tissue destruction through their production of IL-17A (10). However, we previously described that Th17.1 and DP cells are elevated in ACPA positive RA patients compared to ACPA negative patients, suggesting a distinct role for these cells in different RA subtypes (15). Also in other immune diseases, such as Crohn's disease and multiple sclerosis, Th17.1 cells are now considered to be more pathogenic (9, 16). The role of DN cells is not clear, since it has not been previously studied in arthritis.

Further characterization and understanding of which of the CCR6 + memTh subpopulations are predominantly responsible for driving synovial fibroblast activation could aid in developing more targeted

therapies for inflammatory arthritis. Therefore, in this study, we examined the individual contribution of these CCR6 + memTh subpopulations to SF activation and examined ways to regulate their function.

Methods

Subjects

Peripheral blood mononuclear cells (PBMC) from healthy individuals were isolated from buffycoats (Sanquin Bloodbank, Rotterdam, the Netherlands). RA PBMC were isolated from treatment-naïve early RA patients embedded in the Treatment in the Rotterdam Early Arthritic Cohort (tREACH), which all fulfilled the American College of Rheumatology 2010 classification criteria for RA. The study was approved by the Medical Ethics Review Board of Erasmus MC Rotterdam. Relevant clinical characteristics are shown in table S1. Synovial fibroblasts were isolated from established RA patients undergoing joint replacement surgery. Informed consent was obtained from all individuals.

Cell sorting

PBMC were isolated from peripheral blood or buffycoats through a ficoll gradient and stored in liquid nitrogen until use. For sorting, isolated PBMC were first pre-purified for CD4 using magnetic beads (Miltenyi Biotech) and then sorted using monoclonal antibodies against CD4, CD25, CXCR3, CCR4 (BioLegend, San Diego, CA, USA), CD45RO and CCR6 (BD Biosciences, San Diego, CA, USA). All cells were $CD4^+CD45RO^+CD25^{low/int}$, and then further sorted on $CCR6^-CCR4^-CXCR3^+$ (Th1), $CCR6^+CCR4^+CXCR3^-$ (Th17/Th22), $CCR6^+CCR4^-CXCR3^+$ (Th17.1), $CCR6^+CCR4^+CXCR3^+$ (DP) or $CCR6^+CCR4^-CXCR3^-$ (DN).

Cell culture

RASF were grown out of small biopsies from synovial tissue after joint replacement surgery. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS, Gibco, Waltham, MA, USA)) and 100 U/ml penicillin/streptomycin (pen/strep, Lonza, Verviers, Belgium) and used between passage 3 and 8.

Sorted CCR6+ memTh subpopulations were cultured at a density of $2.5x10^4$ cells/ml in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS, 2 mM L-glutamine (Lonza), 100 U/ml pen/strep and 50 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). Where indicated, the CCR6+ Th memory subpopulations were cocultured with $1.0x10^4$ RA synovial fibroblasts (RASF), which were seeded in flat-bottom 96-well plates 24 hours earlier. T cells were stimulated with 300 ng/ml soluble anti-CD3 and 400 ng/ml soluble anti-CD28 (Sanquin, Amsterdam, the Netherlands). For some experiments, the cells were cultured with 100 nM $1,25(OH)_2D_3$ dissolved in 100% ethanol (Leo Pharmaceutical Products, Ballerup, Denmark), 100 µg/ml secukinumab (anti-IL-17A, Novartis, Basel, Switzerland) or 10 µg/ml anti-IFNγ (R&D Systems, Minneapolis, MN, USA). Controls were an equal volume of 100% ethanol (with a

maximum final ethanol concentration of 0,1%) or equal concentrations of corresponding isotype control antibodies, respectively.

Lentiviral vectors and T cell transduction

LVRU6MP plasmids containing scrambled shRNA or four different TBX21-targeting shRNAs were obtained from GeneCopoeia (Rockville, MD, USA). They were amplified in GCI-5α competent cells (GeneCopoeia) and extracted using the PureYield Plasmid Midiprep System (Promega Benelux, Leiden, the Netherlands), both following manufacturer's protocol. LVRU6MP plasmids and the Lenti-PAC HIV Expression Packaging Kit (GeneCopoeia) were used to generate replication-deficient lentiviral vectors in 293T cells (GeneCopoeia) as described in manufacturer's protocol. 293T culture supernatant containing lentiviral vectors was filtered through 0.45 µm polyethersulfone filters and stored at -80°C until use.

For transduction of T cells, CCR6+ Th memory cells ($CD4^+CD45RO^+CCR6^+CD25^{lo/int}$) were sorted and activated with 300 ng/ml soluble anti-CD3 and 400 ng/ml soluble anti-CD28 for two days. Then medium was replaced with either 50% 293T culture supernatant containing relevant lentiviral vectors and 50% normal T cells medium, or 100% T cell medium for mock conditions, both supplemented with 8 ng/ml polybrene (Sigma-Aldrich). T cells were then transduced by centrifuging cells at 1000xg and 32°C for 90 minutes, followed by 4 hours incubation at 37°C and 5% CO2. Then transduction medium was removed and cells were further cultured in normal T cell medium at a density of 0,1x10^4 cells/ml together with 0,5x10^5 autologous irradiated PBMC (50 Gy, using RS320, X-strahl, Surrey, UK) and 50 ng/ml phytohaemaglutinin (PHA). After six days of culture, cells were restimulated with 5 ng/ml IL-2 and cultured for another six days. Then the stably transduced cells were sorted based on MFP expression and cultured with SF as described in the cell culture section.

Flow cytometry

For intracellular cytokine detection by flow cytometry, cultured cells were restimulated for 4 hours using 50 ng/ml phorbol 12-myristate 13 acetate (PMA) (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich) and Golgistop (BD Biosciences). Restimulated cells were stained with Fixable Viability Dye eFluor506 (eBioscience, San Diego, CA, USA) following manufacturer's instructions. For intracellular staining, the cells were fixed with 2% paraformaldehyde in PBS and permeabilized using 0,5% saponin in FACS buffer (PBS with 0,5% BSA and 0,05% NaN3). Monoclonal antibodies against IL-17A, IL-22 (eBioscience) and IFNγ (BioLegend) were then used for staining in permeabilization buffer. Samples were measured on a FACSCantoII Flow Cytometer (BD Biosciences).

ELISA

Concentration of IL-17A, IL-17F, IL-22, IFNγ, GM-CSF, IL-6, IL-8 (all eBioscience), MMP1 and MMP3 (both R&D systems) were measured in culture supernatant using ELISA following manufacturer's protocols.

RT-PCR

RNA was isolated with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), treated with 0.1 U/µl DNAse (Invitrogen) and reverse transcribed into cDNA using 10 U/µl Superscript II (Invitrogen) and random hexamer primers. Primers and probes were designed and chosen using ProbeFinder Software and the Universal Probe library (Roche Applied Science, Indianapolis, IN, USA) and are listed in table S2. Hypoxanthine phosphoribosyltransferase (HPRT) was used to normalize gene expression. RT-PCR was performed with the Viia7 System and analyzed using QuantStudio Real-Time PCR Software version 1.3 (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

To test differences between two or more groups with equal variances, Student's T-test or ANOVA with a Tukey post-test were used, respectively. For groups with unequal variances, Kruskal-Wallis tests were performed with Dunn post-test. Statistical analyses were done using Prism software version 6.01 (GraphPad Software, La Jolla, CA, USA).

Results

CCR6+ memTh subpopulations differ in cytokine production, but not in expression of RORC, in healthy donors and treatment-naïve RA patients

For studying the role and properties of CCR6+ memTh subpopulations, Th17/Th22, Th17.1, double positive (DP) and double negative (DN) cells were sorted from healthy PBMC based on the chemokine receptors CXCR3 and CCR4 (figure 1A). Directly after sorting, Th17/Th22 cells showed a trend towards being the strongest producers of IL-17A, IL-22 and GM-CSF, whereas Th17.1 and DP cells produced the highest levels of IFNγ (figure 1B, upper panel). Furthermore, Th17.1 and DP cells produced significantly more TBX21 than Th17/Th22 cells. Interestingly, RORC was equal in all subpopulations despite the differences in IL-17A expression (figure 1B, upper panel).

Since the phenotype stability of the subpopulations is currently unknown, these sorted cells were cultured for three days under stimulation of anti-CD3 and anti-CD28 and examined for cytokine and transcription factor expression (figure 1B, lower panel). After three days, both Th17/Th22 and DN cells expressed high levels of IL-17A mRNA, although the differences with Th17.1 and DP cells did not reach statistical significance. All subpopulations expressed IL-22, with DP cells producing slightly less than the

Th17/Th22 population. After three days of culture, Th17.1 and DP cells expressed significantly more IFNγ mRNA than Th17/Th22 cells similar to the *ex vivo* expression pattern. The expression pattern of TBX21 after culture matched the expression of IFNγ, with the highest levels in Th17.1 and DP cells and lowest in Th17/Th22 and DN cells (figure 1B, lower panel). The findings on the cytokine expression pattern on the mRNA level were also found on the protein level, both in the percentage of cytokine-expressing cells (figure 1C) and cytokine levels in the culture supernatant (figure 1D). These data suggest that the three-day culture and stimulation of the sorted $CCR6^+$ memTh subpopulations does not significantly alter the profile of the cells based on expression of IL-17A, IFNγ, RORC and T-bet.

Although these data provide information on the healthy properties of the subpopulations, they do not necessarily represent the same phenotype as the subpopulations in inflammatory arthritis where the T cells are abnormally activated. Therefore, the $CCR6^+$ subpopulations were also studied in PBMC from treatment-naïve RA patients. Similar to healthy donors, all four subpopulations were present in the PBMC of these treatment-naïve patients, with the majority being Th17/Th22 cells (figure 2A). Importantly, the subpopulations could also all be distinguished within the synovial fluid mononuclear cells (SFMC) and PBMC of established RA patients (figure 2B). Similar to the findings in healthy cells, all CCR6+ memTh subpopulations expressed equal levels of RORC, whereas IL-17A was predominantly produced by Th17/Th22 cells. Also the expression patterns for IFNγ and TBX21 were similar to the healthy situation, with the highest levels in Th17.1 cells and the lowest in Th17/Th22 cells. These data demonstrate that although all CCR6+ memTh subpopulations express equal levels of RORC, they differ in other parameters such as IL-17A and IFNγ. These properties are similar between healthy donors and treatment-naïve early RA patients.

All CCR6+ subpopulations can activate synovial fibroblasts to create a pro-inflammatory feedforward loop

To determine whether the differences in pro-inflammatory cytokines secreted by the individual CCR6+ memTh subpopulations resulted in different pathogenic potential, the ability of the subpopulations to activate synovial fibroblasts (SF) from patients with rheumatoid arthritis was investigated. CCR6+ memTh subpopulations, but also the classical Th1 cells (CCR6- CXCR3+ CCR4-) as a negative control, were sorted from healthy donors as in figure 1A and subsequently activated and cocultured with SF for three days. When compared to Th1 cells, all CCR6+ subpopulations activated SF as measured by increased IL-6 and IL-8 production (figure 3A). However, there were differences in the extent of activation, with Th17/Th22 cells being more potent inducers of IL-6 and IL-8 than Th17.1 and DP cells. Interestingly, only Th17.1 and DP cells induced MMP1 (figure 3A). The properties of the CCR6+ memTh populations in terms of IL-17A and IFNγ expression did not differ in the coculture with SF compared to the activated CCR6+ memTh populations only cultures in figure 1D, although the overall levels were slightly elevated due to the interaction with SF.

Similar to the CCR6+ memTh subpopulations from healthy individuals, those from treatment-naïve early RA patients also all activated SF upon coculture as demonstrated by the increased levels of IL-6 and IL-8, although this did not reach statistical significance (figure 3B). Interestingly, in contrast to the 'healthy CCR6+ T cells', the CCR6+ memTh subpopulations from RA patients did not differ in their capacity to induce IL-6 and IL-8. Furthermore, where in the healthy situation mainly Th17.1 and DP cells induce MMP1, all subpopulations from RA patients demonstrated a trend towards induction of MMP1 in SF. Similar to healthy individuals, the properties of the subsets in terms of IL-17A and IFNγ expression did not drastically change upon coculture with SF, with Th17/Th22 the highest in IL-17A and the lowest in IFNγ, in contrast to Th17.1 (figure 2 and 3B).

Altogether, these data indicate that all CCR6+ memTh subpopulations from both healthy individuals and RA patients can activate SF. However, there are subtle differences between the healthy and arthritic T cells in terms of which pro-inflammatory factors they predominantly activate in RASF.

All CCR6+ subpopulations use IL-17A to induce IL-6 production by SF

Since all $CCR6^+$ subpopulations are more potent activators of SF than Th1 cells despite their differences in cytokine and transcription factor expression, we next studied which factors play a role in this pro-inflammatory feedforward loop. Previously, we have shown that induction of the pro-inflammatory loop by CCR6+ Th memory cells is mediated by IL-17A (10). However, since not all CCR6+ subpopulations express equal amounts of IL-17A, we first investigated the importance of IL-17A for SF activation in all individual CCR6+ subpopulations. Given the similar properties of the T cell subpopulations from healthy donors and RA patients and the limited availability of cells from RA patients, these functional studies were conducted with healthy donor T cells. Upon treatment with anti-IL-17A the induction of IL-6 by all subpopulations decreased, although this did not reach statistical significance for DP cells (figure 4A). IL-8, MMP1 and MMP3 also showed a decreasing trend upon IL-17A neutralization. The SF activation by DP cells was hardly affected by IL-17A neutralization, suggesting that these cells work via a different mechanism.

One of the features of the pro-inflammatory loop between SF and CCR6+ memTh is that increased activation of the SF leads to increased activation of the Th cells. Interestingly, despite the reduced SF activation upon IL-17A blockade, the T cell derived cytokines IL-17A, IL-22, IFNγ and GM-CSF were not affected by anti-IL-17A treatment (figure 4B). Since IL-17A induction by SF depends on PGE2 (17), this lack of effect could be due to non-responsiveness of PGE2 on the anti-IL-17A treatment. However, PGE2 was significantly inhibited by anti-IL17A in Th17 and DP cells, and showed a trend towards reduction in DN and Th17.1 cells (figure 4C). From these data we conclude that IL-17A blockade reduces SF activation by reducing IL-6 in particular, but that these SF are still capable of further activating the Th cells. This suggests that a mechanism that does not include PGE2 or IL-6 is responsible for the Th cell activation in the SF-Th cell cocultures.

Activation of SF by CCR6+ subpopulations is independent of IFNγ and T-bet

Since IL-17A was mainly responsible for the induction of IL-6 by SF, other cytokines may also play a role in the pro-inflammatory loop between CCR6+ subpopulations and SF. Previous studies have shown that IFNγ suppresses production of MMP1 and MMP3 in IL-1β-stimulated SF, suggesting a modulatory role for this cytokine in the early stages of inflammation (18). Therefore, the role of IFNγ in our system was studied. In contrast to IL-17A blockade, neutralizing IFNγ had no significant effect on the production of IL-6, IL-8, MMP1 and MMP3 (figure 5A). Even though less IFNγ was detected in the culture supernatant, there was no effect on the other T-cell derived cytokines (figure 5B).

Next to IFNγ, also TBX21 showed a distinct pattern of expression between the subsets. Therefore, T-bet, the protein encoded by TBX21, was knocked down using lentiviral shRNA. Due to the low cell numbers that can be obtained for the individual subpopulations, we used the entire CCR6+ Th memory pool for these experiments. In the transduced CCR6+ Th memory cells T-bet expression was decreased by 70% (figure 5C). Importantly, this repression did not induce EOMES, a transcription factor known to take over the role of T-bet (figure 5C). When the T-bet-deficient CCR6+ Th memory cells were cultured together with SF, there was no reduction in fibroblast activation as demonstrated by IL-6, IL-8, MMP1 and MMP3 expression, despite a reduction in IFNγ and a slight increase in IL-17A (figure 5D). Altogether, these data indicate that the activation of SF and induction of the pro-inflammatory loop between SF and CCR6+ Th memory cells are independent of IFNγ and T-bet.

Both SF activation and the pro-inflammatory loop between CCR6+ subpopulations and SF can be inhibited by $1,25(OH)_2D_3$

Previously, we have shown that blocking IL-22 does not affect the pro-inflammatory loop between CCR6+ memTh cells and SF (19), and here we found that IFNγ neutralization has no effect, while IL-17A is partially responsible for SF activation. However, the combined blockade of all pro-inflammatory T cell cytokines may have additional benefits. We have previously shown that CCR6+ memTh cells are highly susceptible to modulation by the active vitamin D metabolite $1,25(OH)_2D_3$ (20-22), leading to decreased production of IL-17A, IL-22 and IFNγ (21, 23, 24). Therefore, this may be a valuable strategy to block these cytokines together and evaluate the effects on SF activation.

Since it is unknown whether the individual CCR6+ memTh subpopulations are susceptible to $1,25(OH)_2D_3$-mediated modulation, we first studied its effects in cultures of individual CCR6+ memTh subpopulations. As expected, the percentage of IL-17A-producing cells decreased in both Th17/Th22 cells and DN cells, the cell types that produce the highest levels of IL-17A. IL-22 was decreased by $1,25(OH)_2D_3$ in all subpopulations, and IFNγ in Th17.1 and DP cells (figure 6A-B). Since this suggests that

all subpopulations can be affected by $1,25(OH)_2D_3$, the compound was added to cocultures of SF and CCR6+ memTh subpopulations. In these cultures, $1,25(OH)_2D_3$ inhibited SF activation by all individual CCR6+ subpopulations, as demonstrated by a decrease in IL-6, IL-8, PGE2 and MMP1, but not MMP3 (figure 6C). $1,25(OH)_2D_3$ also decreased the production of IL-17A, IL-22 and IFNγ similar to the data in cultures of T cells alone, but not GM-CSF (figure 6D). Therefore, we concluded that $1,25(OH)_2D_3$ significantly inhibits the pro-inflammatory loop of all the individual CCR6+ memTh subpopulations cocultured with SF.

Discussion

In this study we have demonstrated that Th17/Th22, Th17.1, DP and DN CCR6+ memTh cells, differentiated based on CCR4 and CXCR3 expression, are individually capable of activating SF and establishing a pro-inflammatory loop which may underlie chronic synovial inflammation. This can be partly suppressed by blocking IL-17A, but not IFNγ or T-bet, and more effective upon the multi-cytokine inhibitor $1,25(OH)_2D_3$.

In recent years there have been numerous studies into Th17 cell subpopulations, including their role in normal immunity and their potential pathogenicity. This led to the discovery that while IL-17A$^+$ T cells can be derived directly *in vitro* through priming them using autologous monocytes pulsed with *Staphylococcus Aureus*, IL-17A$^+$IFNγ$^+$ cells (also named Th17/1 cells) require *Candida Albicans* (25). Furthermore, IL-17A$^+$IFNγ$^+$ cells can also arise when IL-17A$^+$ cells are stimulated with IL-12 and upon exposure to synovial fluid from juvenile idiopathic arthritis (JIA) patients (6, 13, 26). However, for IFNγ$^+$IL-17A$^-$RORC$^+$ (Th17.1) cells and other Th17 cell subpopulations as described in the present study, this ontogeny is less clearly defined. Since the current study shows that based on cytokine and transcription factor expression DN and DP have characteristics in between those of Th17 and Th17.1 cells, it is possible that they represent intermediate cell types that are influenced by local inflammatory conditions. To further characterize these differences and determine whether DN and DP are indeed intermediate cell types or are distinct from Th17 and Th17.1 cells, full transcriptomic and proteomic analysis are required.

In various autoimmune diseases, such as multiple sclerosis, sarcoidosis and Crohn's disease, Th17.1 cells are thought to be especially pathogenic due to their high numbers at the site of inflammation, specific recruitment to the brain or drug resistance (9, 14, 27). Also in juvenile idiopathic arthritis (JIA) these cells are increased in the synovial fluid compared to the peripheral blood (13, 26). However, the present study did not imply a particular pathogenic or regulatory role of Th17.1 cells when it comes to SF activation. Our data suggest that all CCR6+ memTh subpopulations can activate SF, although there are differences between the subpopulations and between healthy individuals and patients with arthritis. Importantly, all subpopulations are present in the synovial fluid of patients with inflammatory arthritis where they can contribute to synovial inflammation. Therefore, targeting all subpopulations may be more relevant in the treatment of patients with persistent synovial inflammation than solely focusing on Th17.1 cells.

Whereas IL-17A blockade partly affected SF activation, IFNγ neutralization had no effect on the interaction between SF and CCR6+ memTh subpopulations. This is contrast with previous findings that IFNγ suppresses MMP1 and MMP3 upon IL-1β stimulation (18). This may be an effect specific for IL-1β-induced MMP production, since the interaction between CCR6+ memTh cells is largely independent of IL-1β (17), but could also be because the interplay between two cell types is more complex than just cytokine-mediated stimulation of one cell type. Interestingly, IFNγ has even been implicated to be protective in autoimmune inflammation and collagen-induced arthritis (28). The data in this study suggest that this protective effect of IFNγ is not mediated via inhibition of fibroblast activation, since neutralization did not aggravate the inflammatory response. Overall, our data show that Th17.1 cells are not specifically pathogenic in terms of SF activation via IFNγ. Although the role of GM-CSF was not investigated in the present study, the finding that it was not inhibited by $1,25(OH)_2D_3$ whereas other inflammatory mediators were inhibited suggests a limited role in regulating IL-6, IL-8 and MMP1 in SF. A potential effect of GM-CSF on MMP3 needs further investigation. However, in contrast to SF activation, IFNγ and GM-CSF are able to perpetuate inflammation through monocyte activation (29, 30).

Since IL-17A, and also TNFα (21), is only partially responsible for the pro-inflammatory loop between SF and CCR6+ memTh populations, there must be another mechanism through which SF activate T cells aside from soluble factors. Th17 cells are known to adhere to collagen via CD49b and thereby stimulate joint damage (31, 32), suggesting that adhesive properties of these cells may be important for their pathogenic functions. Indeed, it has been demonstrated that cell-cell contact is also required for the pro-inflammatory interaction between SF and T cells, probably via VCAM-1 and ICAM-1 (33). Further research should study the importance of adhesion molecules in the pro-inflammatory feedforward loop and identify which molecules are crucial in the interaction. This could provide clues on how to fully suppress SF activation and the subsequent persistent synovial inflammation.

An interesting therapeutic option would be to utilize a broader T cell cytokine inhibitor, as demonstrated by the use of $1,25(OH)_2D_3$ in our study. This active vitamin D metabolite has been shown to suppress multiple Th17 cytokines and Th17 pathogenicity (21, 22), and here we have shown that the individual CCR6+ memTh subpopulations are susceptible for modulation by $1,25(OH)_2D_3$. Furthermore, $1,25(OH)_2D_3$ inhibits activation of both SF and CCR6+ memTh subpopulations in coculture. There are several hypotheses that can explain the higher efficacy of $1,25(OH)_2D_3$ compared to single blockade of IL-17A, IL-22 or IFNγ. First of all, blocking each cytokine separately may result in other cytokines taking over. Since $1,25(OH)_2D_3$ blocks all T cell-derived pro-inflammatory cytokines at once, this is no longer possible. Another possibility is that $1,25(OH)_2D_3$ not only affects the pro-inflammatory cytokines, but also the cell-cell interaction. For example, it has been shown that $1,25(OH)_2D_3$ inhibits VCAM-1 in endothelial cells and VLA-4 in various cell lines (34, 35). Furthermore, we have previously shown that $1,25(OH)_2D_3$ induces a regulatory phenotype in CCR6+ memTh cells which could potentially further decrease inflammation in the cocultures with RASF (23). Finally, it is also possible that $1,25(OH)_2D_3$ modulates SF directly (36). However, in our hands the direct effect of $1,25(OH)_2D_3$ on SF is always minimal compared to the effects seen in the SF-Th cocultures (data not shown), suggesting that the main mode of action of $1,25(OH)_2D_3$

is through T cell modulation. Since $1,25(OH)_2D_3$ is not a clinically applicable therapeutic due to severe side effects, the molecular mechanism underlying these changes should be investigated to find new therapeutic targets. Another possibility is the use of specific Jak inhibitors that are current being started in the treatment of inflammatory arthritis, which can also target multiple cytokine pathways together.

Finally, since all CCR6+ memTh express RORC and therefore by the classical definition considered Th17 cells, their pathogenicity may also be reduced by inhibiting this transcription factor. In rat models for RA pharmacological inhibition of RORC indeed reduced joint inflammation, suggesting it could be effective in the treatment of inflammatory arthritis (37). However, therapeutic safety and efficacy still needs to be investigated.

Conclusions

In conclusion, human CCR6+ memTh cells form a heterogeneous population, including Th17/Th22, DP and Th17.1 cells that express RORC but differ in T-bet, IFNγ and IL-17A expression. All individual human CCR6+ memTh subpopulations are more potent in activating synovial fibroblasts than classical Th1 cells in an IFNγ-independent way. Furthermore, our data suggest that IL-17A is not dominant in this T cell-RASF activation loop but that a multiple T cell cytokine inhibitor, such as 1,25(OH)2D3, but potentially also specific Jak inhibitors, is able to suppress CCR6+ memTh subpopulation-driven RASF activation. These novel data suggest that although the individual differences between the CCR6+ memTh subpopulations, there is an overlapping mechanism responsible to drive synovial activation. Therefore, future research and therapeutic interventions should focus on identifying this general pathogenic mechanisms between CCR6+ memTh subpopulations and SF interaction.

Declarations

Ethics approval and consent to participate

The tREACH trial was approved by the Medical Ethics Review Board of Erasmus MC Rotterdam. Informed consent was obtained from all patients in the tREACH trial and patients undergoing joint replacement surgery to use their tissues.

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

WD contributed to study design, performed experiments, analyzed data and wrote the manuscript. HdB contributed to study design, performed experiments, analyzed data and critically revised the manuscript. SP and JvH performed experiments and analyzed data. ND performed experiments. EC contributed to study design and critically revised the manuscript. EL designed and supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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Supplemental Table

Supplemental Tables are not available with this version

Figures

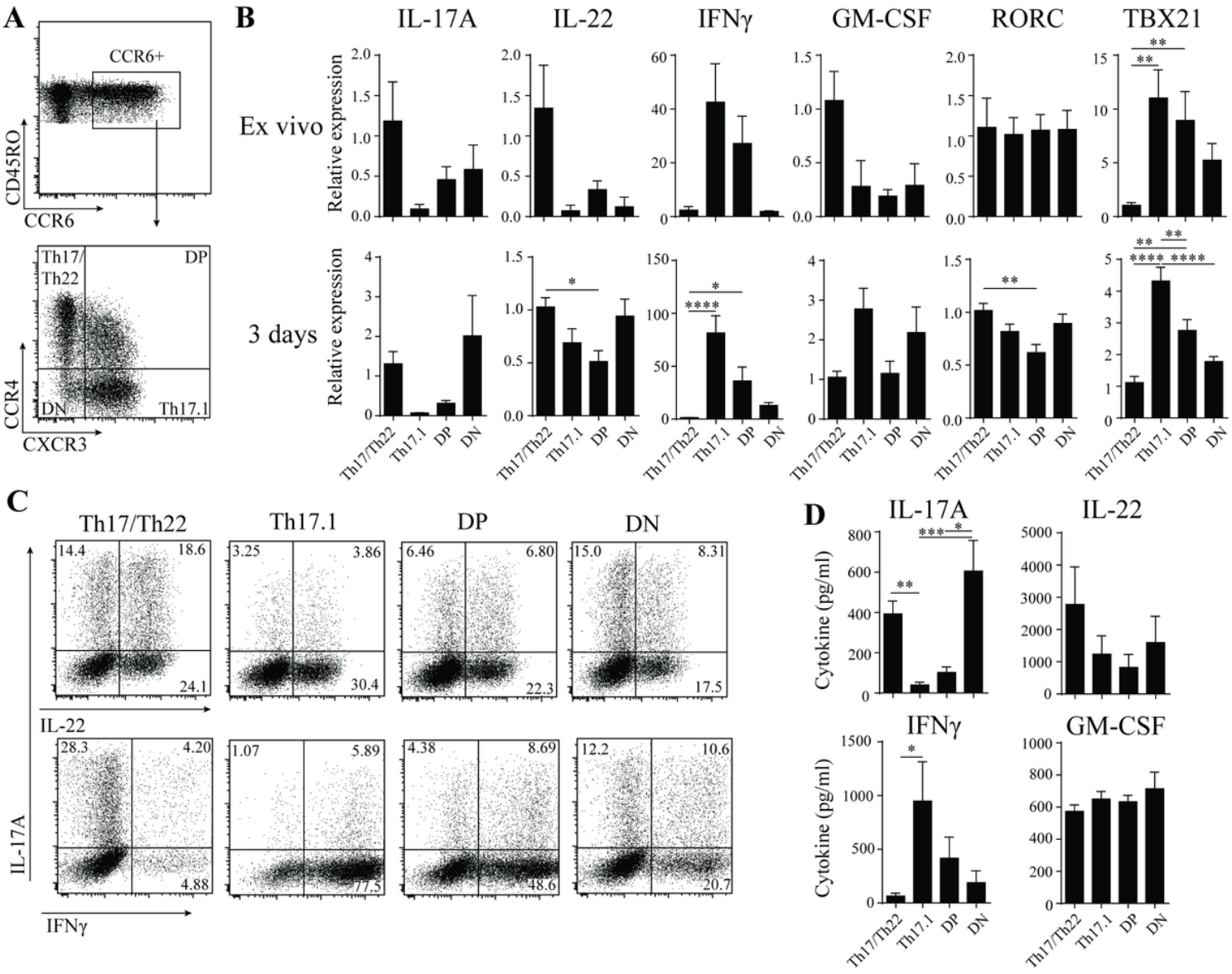

Figure 1

CCR6+ Th memory subpopulations differ in cytokine production, but not in RORC expression. (A) Gating strategy for the four subpopulations. (B) Gene expression of sorted cells, directly ex vivo (upper panel) or after three days of stimulation with anti-CD3 and anti-CD28 (lower panel) is determined using RT-PCR. (C) Representative flow cytometry plot of IL-17A, IL-22 and IFNγ expression after three days of stimulation from one healthy donor. (D) Production of IL-17A, IL-22, IFNγ and GM-CSF after three days of stimulation as measured by ELISA. Data represent mean ± SEM of n=3-8 healthy controls of at least 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

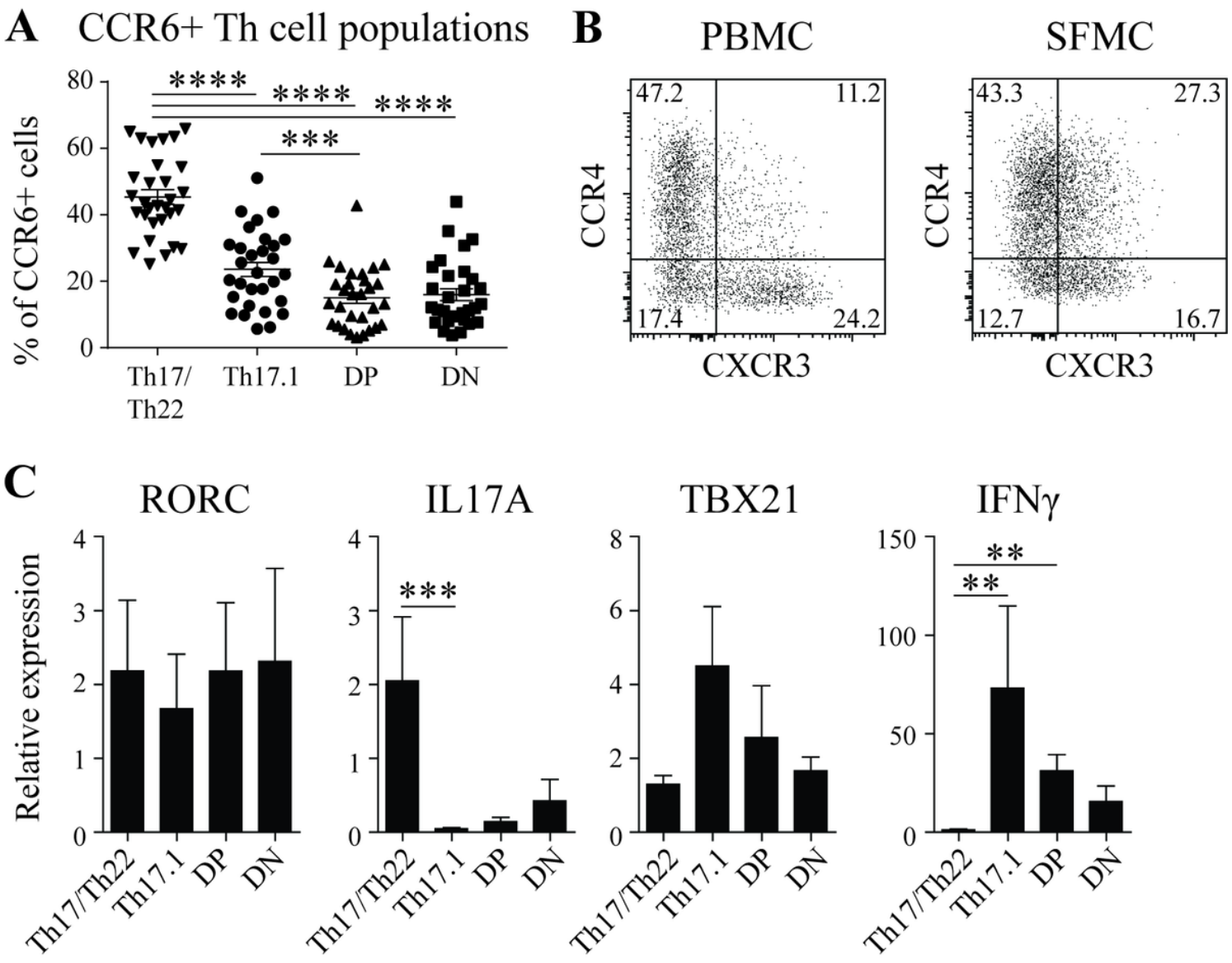

Figure 2

CCR6+ memTh subpopulations from treatment-naïve RA patients have similar properties as those from healthy controls. (A) Percentage of Th17, Th17.1, DP and DN cells in 32 treatment-naïve early RA patients. (B) Representative flow cytometric plot of CCR6+ memTh subpopulations in paired PBMC and SFMC from an established RA patient. (C) Gene expression after three days of culture upon stimulation of anti-CD3 and anti-CD28 as measured by RT-PCR, representing n=5-10 treatment-naïve early RA patients (mean ± SEM). **p<0.01, ***p<0.001, ****p<0.0001.

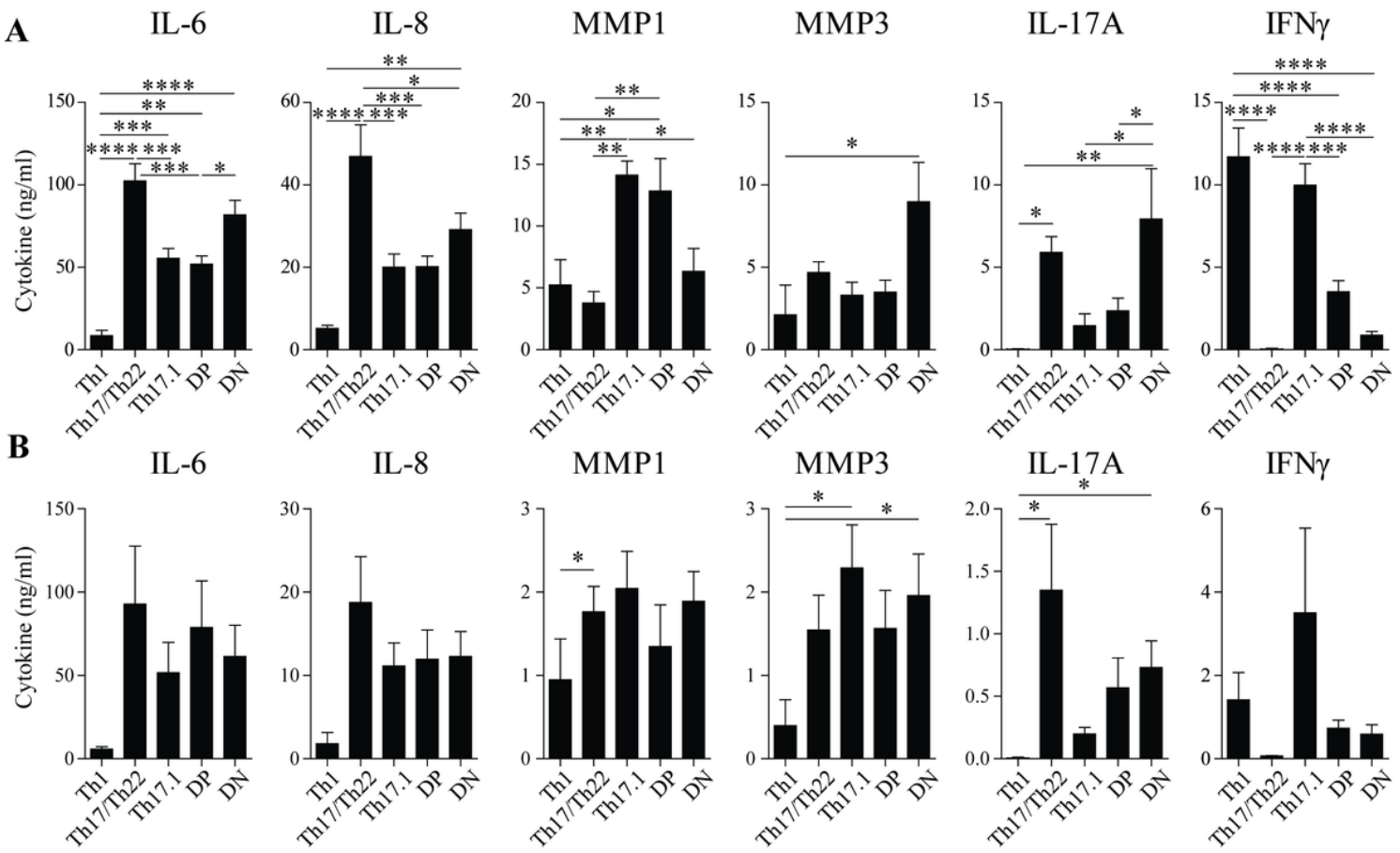

Figure 3

All CCR6+ subpopulation from healthy controls and RA patients activate SF. Sorted CCR6+ subpopulations from healthy donors (A, 25.000 T cells) or treatment-naïve early RA patients (B, 4000-10000 T cells) were cultured for three days together with SF upon stimulation with anti-CD3 and anti-CD28. Cytokines were measured in the culture supernatant on day 3 using ELISA. Data are shown as mean ±SEM of n=6 healthy controls or n=5 RA patients and representative of at least 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

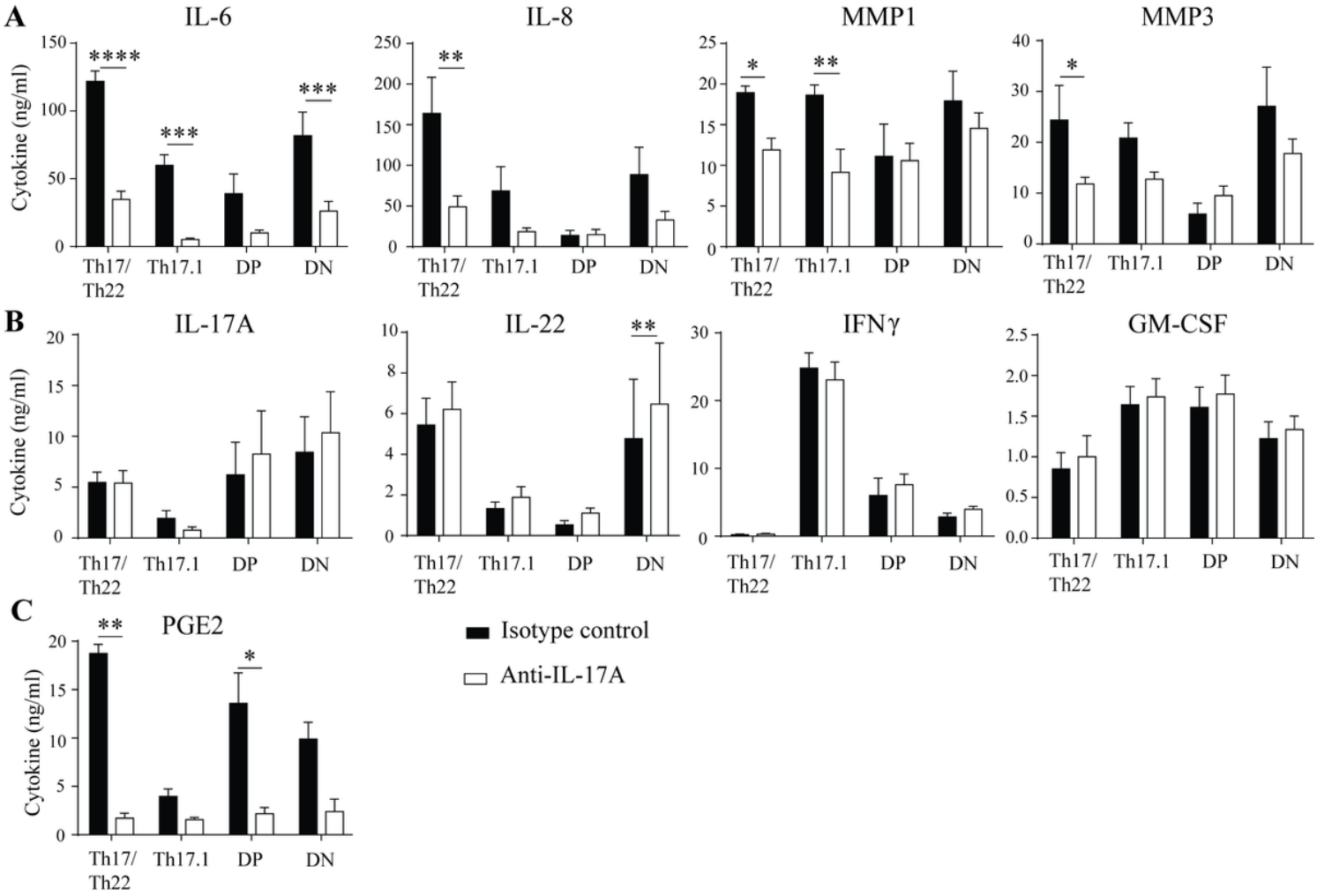

Figure 4

IL-17A plays a role in SF activation, but not in induction of a pro-inflammatory loop. The CCR6+ subpopulations were sorted from healthy controls as in figure 1A and cultured as in figure 2, with the addition of 100 µg/ml secukinumab or an isotype control. ELISA was used to measure SF-derived mediators (A), T-cell derived cytokines (B) and PGE2 (C) in the culture supernatant on day 3. Data represent mean ±SEM from n=6 subjects, pooled from 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

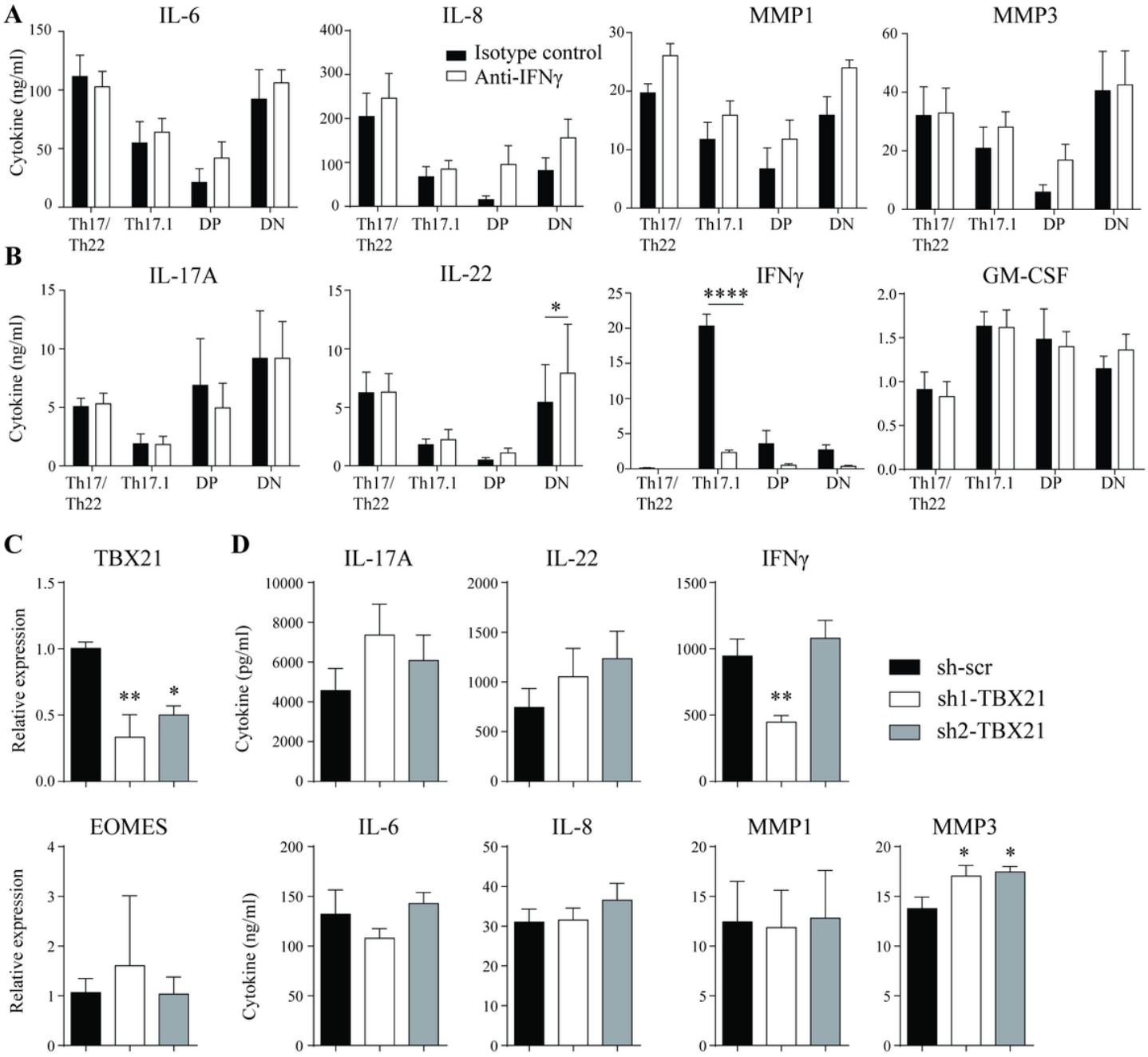

Figure 5

SF activation is independent of IFNγ and TBX21. (A and B) CCR6+ subpopulations were cultured and analyzed as in figure 4, but with 10μg/ml neutralizing IFNγ antibody or isotype control. (C) Total CCR6+ Th memory cells were transduced with a scrambled shRNA (sh-scr) or two different TBX21 shRNA (sh1-TBX21 and sh2-TBX21). Stably transduced CCR6+ Th memory cells were cultured for three days with SF under stimulation of anti-CD3 and anti-CD28. Expression of TBX21 and EOMES was measured using RT-PCR on day 3. (D) Cytokine expression in culture supernatant from (C) was determined after three days using ELISA. Data represent mean ±SEM from n=6 healthy controls, pooled from 2 independent experiments. *p<0.05, **p<0.01, ****p<0.0001. In figures C and D, *, ** and *** represent differences compared with scrambled shRNA.

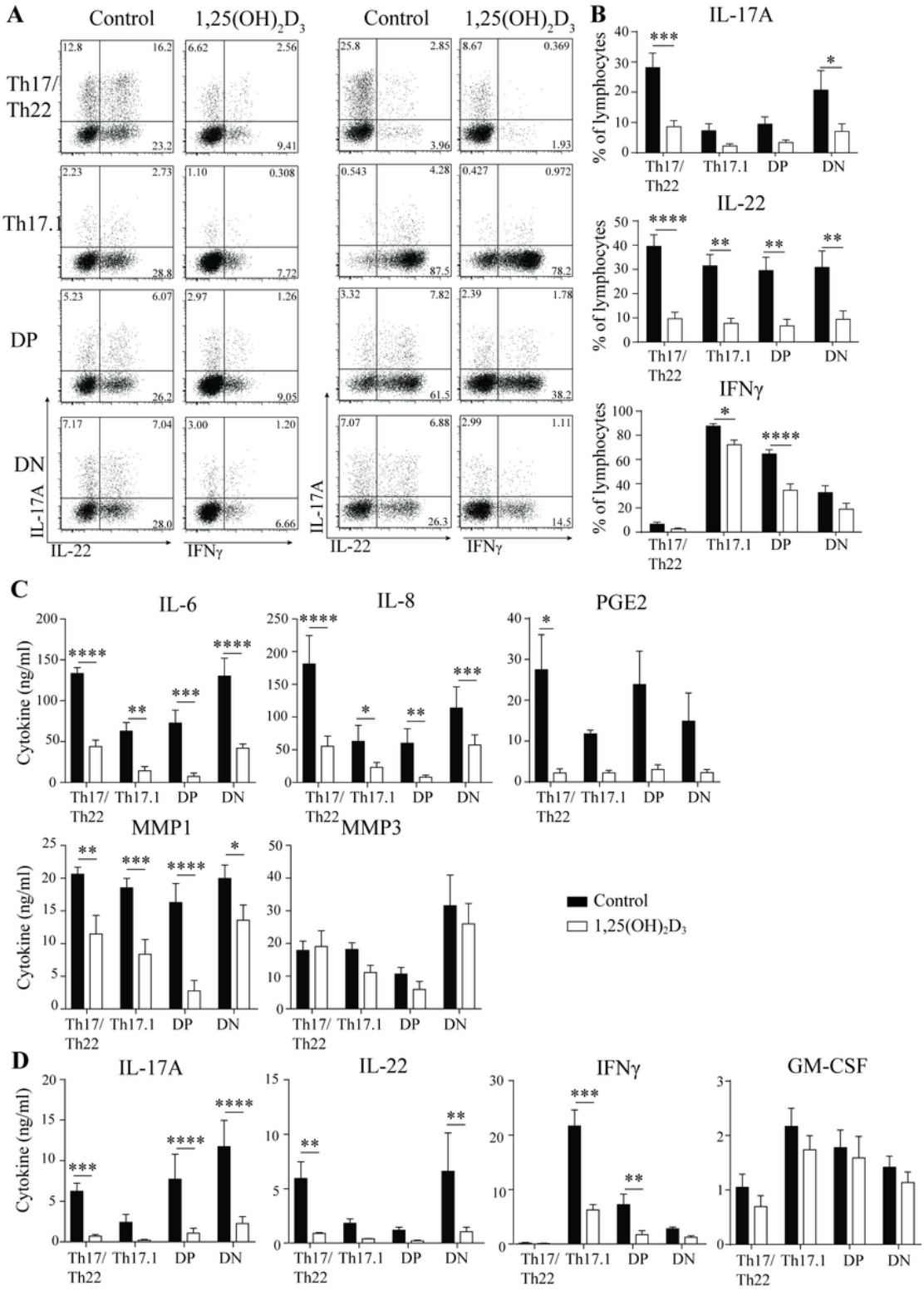

Figure 6

1,25(OH)2D3 can reduce SF activation and the pro-inflammatory loop. CCR6+ subpopulations were cultured with SF for three days with stimulation from anti-CD3 and anti-CD28 in the presence of 100 nM 1,25(OH)2D3. (A-B) Representative flow cytometry plots and quantification of 6 healthy donors displaying IL-17A, IL-22 and IFNγ-producing cells after three days of culture. (C-D) Expression levels of cytokines,

MMPs and PGE2 in culture supernatant on day 3. Data show mean ±SEM from n=6 healthy controls, representative of at least 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.