Advances in Rheumatology

RESEARCH

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Mesenchymal stem cells modulate IL-17 and IL-9 production induced by Th17-inducing cytokine conditions in autoimmune arthritis: an explorative analysis

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Abstract

Background The importance of proinflammatory T-cells and their cytokine production in patients with autoimmune arthritis has been widely described. Due to their immunomodulatory properties, mesenchymal stem cells (MSCs) have come into focus as a potential therapeutic concept. The aim of this study was to investigate the influence of MSCs on the phenotype, cytokine profile, and functionality of naive and non-naive $CD4^+$ T-cells from healthy donors (HD) and patients with autoimmune arthritis under Th17-cytokine polarizing conditions in an explorative way using a transwell system prohibiting any cell–cell-contact.

Methods Magnetically isolated naive and non-naive $CD4^+$ T-cells were stimulated under Th17-polarizing proinflammatory cytokine conditions in presence and absence of bone marrow derived mesenchymal stromal cells (MSCs). After an incubation period of 6 days, the proportions of the T-cell subpopulations TEMRA ($CD45RA^+CD27^-$), memory ($CD45RA^-CD27^+$), effector ($CD45RA^-CD27^-$) and naive cells ($CD45RA^+CD27^+$) were determined. Quantitative immunofluorescence intensity was used as a measure for IL-9, IL-17 and IFN-γ production in each subpopulation**.**

Results In isolated naive $CD4^+$ T-cells from HD and patients, MSCs suppressed the differentiation of naive towards an effector phenotype while memory and naive cells showed higher percentages in culture with MSCs. In patients, MSCs significantly decreased the proportion of IL-9 and IL-17 producing effector T-cells. MSCs also reduced IFN-γ production in the naive and memory phenotype from HD.

Conclusion The results of the study indicate significant immunomodulatory properties of MSCs, as under Th17-polarizing conditions MSCs are still able to control T-cell differentiation and proinflammatory cytokine production in both HD and patients with autoimmune arthritis.

Keywords Mesenchymal stem cell, Arthritis, T lymphocytes

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Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem (or stromal) cells with the capacity of self-renewal and differentiation potential towards several mesodermal linages such as osteogenic, chondrogenic and adipogenic differentiation (more citations) [1]. The anti-inflammatory role and immune-modulating properties of MSCs have been shown in several studies and their potential is being tested and discussed already in clinical trials including indications for e.g. graft-versus-host disease and even COVID19 pneumonia [2–6] Specifically, inhibition of the effector T-cell activation, in both $CD4^+$ and $CD8^+$ T-cells, has been associated to an increase of proportions of regulatory T-cells (Treg). Moreover, reduction in the number of IL-17 and IFN-γ producing T-cells were observed in the presence of MSCs [1, 7]. A shift from Th1 to Th2 cells demonstrated by an increase in the levels of IL-4 has been observed upon treatment with MSCs [8]. Although, the mechanism how MSCs modulate T-cells has been still not elucidated, cell–cell contact and some soluble factors such as nitric oxide, IL-27, TGF-beta, human leukocyte antigen G, and prostaglandin E_2 may be the key factors in the process [4, 9–11]. Based on their potential regenerative and immunomodulatory properties, MSCs have been proposed as a possible therapeutic approach not only for the treatment of autoimmune-associated diseases [12–14] such as rheumatoid arthritis (RA) [15], systemic lupus erythematosus (SLE) [16], and ankylosing spondylitis (AS) [17], in graft-versus-host-disease and COVID19 infection as discussed above, but also in tissue engineering [18].

Interleukin-9 (IL-9) helps to eliminate the pathogen in parasitic infections and promote the maintenance of a tolerant environment in skin transplants [8]. IL-9 is produced by mast cells [19] and Th2 cells [20], whereas IL-9 may be also derived from natural killer cells (NK) [21], Th9 [22], Th17 cells [23], Tregs [24] and $CD8^+$ T-cells [25]. In the presence of TGF-β, IL-9 may induce differentiation of naive $CD4^+$ T-cells towards Th17 cells, while IL-9 also affects thymus-derived natural Tregs (nTregs) and enhances their suppressive function in vitro [26]. However, IL-9 has been associated with allergies and autoimmunity [19, 27]. IL-9 and Th9 cells are overexpressed in synovial tissue while proportion of Th9 cells are increased in peripheral blood from RA patients [28]. Interestingly, some studies showed that MSCs decrease IL-9 production by reducing the percentage of pro-inflammatory T-cells and increased levels of Treg cells [29]. However, if MSCs are co-cultured with $CD4^+$ T-cells under Th17-polarizing conditions, they seem to have a pronounced immunosuppressive effect on Th1 and Th17 cells as already mentioned. After T-cell polarization has already been induced, MSCs appear to suppress Th1 differentiation, but stimulate Th17 differentiation. This indicates that there might be positive and negative effects on the T-cell modulation by therapeutically used MSCs depending on the existing inflammatory conditions [30]. Other results confirm proinflammatory effects of MSCs on T-cells after cell–cell contact associated with an increased IL-17 production in $CD4^+CD45RO^+$ memory cells [31]. Ultimately, the immune response seems to be both promoted and inhibited by MSCs and seems to depend on the dynamics of the inflammation, the activation state of the immune system, current inflammatory cytokines and the effects of immunosuppressors [32]. Nevertheless, there remain some significant inconsistencies and a general lack of information regarding a number of effects that MSCs have on T-cell subpopulations [1].

Recently, in a study of Heim et al. we could show that Th9- and Th17- inducing cytokine conditions mimicking autoimmune inflammation in RA may have similar stimulatory effects regarding polarization of naive and non-naive T-cells into Th9 or Th17 cells [33]. From our point of view, the influence on the polarization of naive and non-naive T-cells in Th9 and Th17 cells by MSC might be a possible therapeutic approach. Therefore, the aim of this study was to investigate the influence of MSCs on the phenotype, cytokine profile, and functionality of naive and non-naive $CD4^+$ T-cells from HD and patients with autoimmune arthritis under Th17-cytokine polarizing conditions in an explorative way using a transwell system prohibiting any cell–cell-contact. This study provides insights for the potential prophylactic use of MSCs in the treatment of T-cell dysbalance found in autoimmune arthritis.

Materials and methods

Patients and healthy donors (HD)

Blood samples of 4 volunteers (37 ± 9 years) with autoimmune arthritis (2 patients with rheumatoid arthritis (RA), 1 patient with spondylarthritis (SA) and 1 patient with systemic lupus erythematosus (SLE) and significant polyarthritis and 4 age- and gender-matched healthy donors (HD) were taken at the Department of Internal Medicine II, Rheumatology and Clinical Immunology University Hospital Würzburg (Table 1). Patients meeting the ACR criteria for RA, SLICC criteria for SLE and ASDAS criteria for AS were included [34–36]. Blood samples from HD were randomly taken from immunologically healthy volunteers without history of autoimmune disease, malignancy, allergy, administration of blood products, monogenetic disorders, immunodeficiency or any infections in the last four weeks and gender matched to the study group.

Table 1 Patient characteristics

	Patient 1	Patient 2	Patient 3	Patient 4
Age (y)	60.8	34.4	23.7	29.8
Sex (m/f)	f	f	f	m
Diagnosis	Seropositive RA	Seropositive RA	SLE polyarthritis	AS
Disease duration (y)	31	15	5	7
DAS28	3.50	3.35	–	–
BASDAI	–	–	–	2.3
BASMI	–	–	–	2
SLEDAI	–	–	12	–
ESR (1 h)	30 mm	4 mm	54 mm	4 mm
CRP (mg/dl)	0.25	0.01	0.04	0.31
Leukocytes (n*1000/µl)	9.9	6.7	6.4	12.8
Remission (yes/no)	No	No	No	Yes
Methotrexate	10 mg 1x/week	–	–	–
Leflunomide	20 mg 1–0-0	–	–	–
Prednisolone	40 mg 1–0-0	7.5 mg 1–0–0	40 mg 1–0–0	–
Infliximab	–	–	–	400 mg 1 × /8 weeks
Mycophenolate mofetil	–	–	500 mg 2–0–2	–
Etanercept	–	50 mg 1x/week	–	–

Table shows age in years (y); sex—male/female (m/f); Diagnosis (RA = rheumatoid arthritis, SLE = systemic lupus erythematosus, AS = ankylosing spondylitis); Years since initial diagnosis; Disease Activity Score 28 (DAS28); Bath Ankylosing Spondylitis Disease Activity Index (BASDAI); Bath Ankylosing Spondylitis Metrology Index (BASMI); Systemic Lupus Erythematosus Disease Activity Index (SLEDAI); Erythrocyte sedimentation rate (ESR) after one hour (1 h); C-reactive protein (CRP) in mg/dl; Leukocytes in n*1000/µl; Medication at the time of blood sample collection (MTX = methotrexate, LEF = leflunomide, PRE = prednisolone, ETA = etanercept, MYC = mycophenolate mofetil, INF = infliximab); Remission yes/no (patient in remission phase yes or no). A DAS28 < 2.6 was defined as remission for patients 1 and 2. Patient 3 showed an increased disease activity corresponding to a total of 12 points in the SLEDAI. In patient 4, a remission of the underlying disease could be assumed on the basis of low numerical values in the BASDAI and BASMI

The study was approved by the local ethics committee of the Medical Faculty of the University Hospital of Würzburg (No. 123/14) and performed in accordance with the Declaration of Helsinki 1967 and its later amendments and the Medical Research Involving Human Subjects Act (WMO).

Exclusion criteria were malignancies, congenital anomalies, syndromes, immunodeficiencies, clinically relevant infections in the last eight weeks and vaccinations in the last four weeks. Written informed consent was given from patients, HD and MSC donors.

Mesenchymal stem cells (MSC)

MSCs were harvested from spongy bone of the acetabulum during orthopedic hip surgery for the treatment of osteoarthritis of 2 otherwise HD and expanded in vitro [37]. MSCs of both donors were in passage 1 at the beginning of the co-culture. Patients with malignancies, femoral head necrosis, femoral head trauma, congenital anomalies, syndromes, immune defects, clinically relevant infections in the last eight weeks and autoimmune inflammatory rheumatic diseases were excluded from the study (Table 2).

Table 2 MSC healthy donor characteristics

	MSC 1	MSC 2
Age (y)	48.9	72.2
Sex (m/f)	m	m
Diagnosis	Coxarthrosis	Coxarthrosis
Origin MSC	Acetabulum	Acetabulum
CRP (mg/dl)	1.6	0.2
Leukocytes (n*1000/µl)	7.7	6.5
Dabigatran	110 mg 0–0–2–0	–
Pantoprazol	20 mg 1–0–0–0	–
Paracetamol	500 mg 2–2–2–0	–
Diclofenac	75 mg 1–0–1–0	–
Levetiracetam	–	500 mg 1–0-1–0
Lorazepam	–	1 mg 0–0–1–0
Simvastatin	–	10 mg 0–1–0–0
Tromcardin® forte	–	0–2–0–0
Perindopril	–	4 mg 0–0–1/2–0

Table shows age in years (y); sex—male/female (m/f); diagnosis; origin of MSCs; c-reactive protein (CRP) in mg/dl; leukocytes in n*1000/µl; medication (in mg) at the time of harvesting MSCs

Naive and non-naive cell sorting

To isolate naive and non-naive $CD4^+$ T-cells, two magnetic-bead-based isolation kits were combined. By a first step, $CD4^+$ T-cells were isolated, in a second step, naive and non-naive cells were separated from the $CD4^+$ T-cell fraction.

Step 1 (Isolation of $CD4^+$ T-cells): $CD4^+$ cells were isolated from peripheral blood mononuclear cells (PBMCs) from HD and patients using the *$CD4^+$ T Cell Isolation Kit human.*

Step 2 (Isolation of Naive and Non-Naive *$CD4^+$ T-cells*): Naive (depleted) and non-naive $CD4^+$ (retained) fractions were obtained using the *Naive $CD4^+$ T Cell Isolation Kit II human.*

Both isolation steps were performed according to the manufacturers' instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). A purity > 90% of the positive fractions was evaluated by flow cytometry. Non-$CD4^+$ T-cells were irradiated (30 Gy) and used as autologous antigen presenting cells (feeder cells).

Co-culture MSC and T-cells

For co-culturing MSCs and T-cell subpopulations, adherent MSCs were removed from the cell culture surfaces by using trypsin/EDTA (1x) solution (PAA Laboratories GmbH, Pasching, Austria) following treatment with cell culture media containing 10% FCS to stop the reaction. To examine the effect of the soluble factors in the modulation of the T-cell response by MSCs, naive or non-naive $CD4^+$ T-cells (2.5×10^5 cells/well) together with feeder cells (5×10^4) were seeded in the upper part of the Transwell system (Greiner Bio-one GmbH, Frickenhausen, Germany), while MSCs (0.25×10^5 cells/well) were seeded on the bottom. Wells with direct cell-to-cell contact between MSCs and T-cells were not included in the study. For Th17-inducing cytokine conditions, wells were stimulated under a Th17 cytokine cocktail consisting of IL-1β (10 ng/mL), IL-6 (20 ng/mL), TGF-β (5 ng/mL), IL-23 (100 ng/mL), anti-CD3/anti-CD28 (1 µg/mL) for 6 days at 37 °C. Wells containing no MSCs were used as control.

Flow cytometry analysis

The cytokine production profile on $CD4^+$ naive and non-naive T-cells treated in the presence or absence of MSCs was determined upon stimulation with phorbol 12-myristate 13-acetate (PMA) (15 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml) for 3 h at 37 °C. After incubation, expression of CD45RA and CD27 on naive and non-naive cells were determined by using monoclonal antibodies labeled with brilliant violet 421 (BV421, BioLegend, San Diego, USA) and CD27, labeled with phycoerythrin-cynanine7 (PE-Cy7, BioLegend, San Diego, USA) respectively. According to the expression of CD45RA and CD27, subpopulations were characterized as terminal effector memory cell re-expressing RA (TEMRA) ($CD45RA^+CD27^-$), memory ($CD45RA^-CD27^+$), effector ($CD45RA^-CD27^-$) and naive cells ($CD45RA^+CD27^+$) [38].

Intracellular cytokine production of IL-9 and IL-17 was determined after treatment with fixation and permeabilization buffer (Biolegend, San Diego, USA) using IL-9 (PE, BD Biosciences, Franklin Lakes, USA) and IL-17 (Alexa Fluor 700, BioLegend, San Diego, USA) monoclonal fluorescent labeled antibodies. Surface marker and cytokine production was evaluated by flow cytometry using FACSCanto II, BD. Data analysis was performed using FACSDiva software V6 (BD, San Jose, CA). Absolute counts of T-cell subpopulations were calculated from total lymphocyte counts in cell cultures and from gated events by flow cytometric analysis.

Statistics

Statistical evaluation was performed non-parametrically using the Mann–Whitney U test. For calculation of the data sets IBM SPSS Statistics Version 22 (Armonk, New York, USA) was used. A $p \leq 0.05$ was considered statistically significant.

Results

MSCs preserved the naive and memory T-cell phenotype on $CD4^+$ T-cells

Distribution of the naive ($CD45RA^+CD27^+$), memory ($CD45RA^+CD27^-$), effector ($CD45RA^-CD27^-$), and TEMRA ($CD45RA^+CD27^-$) subpopulation after stimulation with a Th17 cytokine cocktail in the presence or absence of MSCs was evaluated in both $CD4^+$ naive and non-naive isolated T-cells from patients and HD. The proportions of naive and memory cells in the naive fraction in both, patients and HD, in the presence of MSCs was higher compared to treated cells in the absence of MSCs (Fig. 1, Table 3). After culture of previously naive $CD4^+$ T cells the proportion of effectors was significantly reduced in the presence of MSCs. Similar profile was observed in the non-naive isolated cells. In this fraction MSCs prevented the differentiation of memory into effector cells as well (Fig. 1, Table 4).

Induction of IFN-γ, IL-17 and IL-9 under Th17 polarizing conditions

Intracellular IFN-γ, IL-17, and IL-9 production was obtained in both, naive and non-naive $CD4^+$ T-cells after stimulation under Th17-polarizing cytokine cocktail in vitro (Fig. 2). Cytokine production in the previously naive fraction was mainly carried out by memory and effector cells, whereas in the previously non-naive

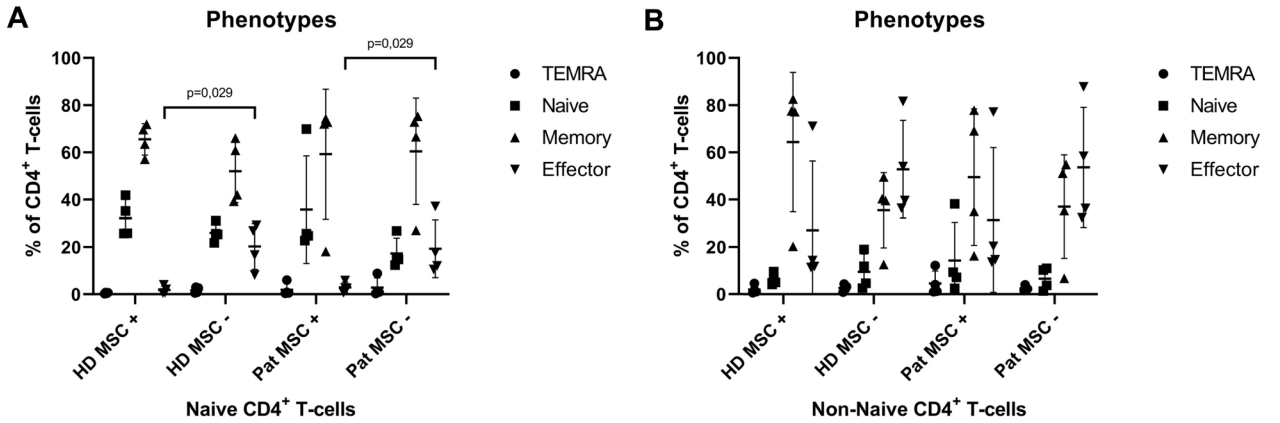

Fig. 1 Dot plots of different phenotypes (TEMRA, Naive, Memory and Effector T-cells) in the naive (**A**) and non-naive (**B**) $CD4^+$ T-cell fraction after 6 days of Th17 stimulation in presence/absence (MSC+/MSC−) of MSCs; HD = healthy donors; Pat = patients; p-values are indicated (Mann–Whitney-U-Test)

Table 3 Phenotyping of naive $CD4^+$ T-cells from healthy donors (HD) and patients (Pat) with/without MSCs

	HD		Pat	
	MSCs	**No MSCs**	**MSCs**	**No MSCs**
TEMRA $CD45RA^+CD27^-$				
%	0.5 ± 0.3 (0.6; 0.2–0.8)[a]	1.8 ± 1.3 (1.8; 0.5–3.0)	2.0 ± 2.7 (0.7; 0.4–6.0)	2.9 ± 4.0 (1.2; 0.3–8.9)
Absolute count	150 ± 90 (180; 60–240)	540 ± 390 (540; 150–900)	600 ± 810 (210; 120–1800)	870 ± 1200 (360; 90–2670)
Naive $CD45RA^+CD27^+$				
%	32.2 ± 7.8 (30.6; 25.8–41.9)	26.0 ± 3.8 (25.6; 21.8–31.1)	35.8 ± 22.8 (25.3; 22.8–69.9)	17.3 ± 6.5 (15.0; 12.4–26.9)
Absolute count	9660 ± 2340 (9180; 7740–12,570)	7800 ± 1140 (7680; 6540–9330)	10,740 ± 6840 (7590; 6840–20,970)	5190 ± 1950 (4500; 3720–8070)
Memory $CD45RA^+CD27^+$				
%	65.5 ± 6.6 (66.6; 57.1–71.9)	52.1 ± 13.4 (51.5; 39.3–66.0)	59.3 ± 27.5 (72.4; 18.1–74.3)	60.5 ± 22.6 (69.8; 27.1–75.3)
Absolute count	19,650 ± 1980 (19,980; 17,130–21,570)	15,630 ± 4020 (15,450; 11,790–19,800)	17,790 ± 8250 (21,720; 5430–22,290)	18,150 ± 6780 (20,940; 8130–22,590)
Effector $CD45RA^-CD27^-$				
%	1.8 ± 1.6 (1.4; 0.5–4.0)	20.3 ± 9.7 (21.8; 8.2–29.3)[a]	2.9 ± 2.2 (2.6; 0.6–5.9)	19.3 ± 12.2 (14.9; 10.5–37.1)[b]
Absolute count	540 ± 480 (420; 150–1200)	6090 ± 2910 (6540; 2460–8790)	870 ± 660 (780; 180–1770)	5790 ± 3660 (4470; 3150–11,130)

Phenotype switch of isolated naive $CD4^+$ T-cells from healthy donors (HD) and patients (Pat) in presence/absence of MSCs after 6 days of Th17 stimulation. Average percentages and absolute count of the different phenotypes of all $CD4^+$ T-cells are shown; Values are shown as follows: mean ± standard deviation (median; minimum – maximum)

[a] Differences between Effector T-cells in HD with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

[b] Differences between Effector T-cells in Pat with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

There were no significant differences between other phenotypes in Pat or HD with MSCs vs. without MSCs (Mann–Whitney-U-Test)

fraction effector cells mainly produced IFN-γ, IL-17, and IL-9. No significant differences were observed between patients and HD (Fig. 2). We also demonstrated that IL-9 can be induced by stimulation with Th17-specific stimuli, such as IL-1β, IL-6, TGF-β, IL-23, and anti-CD3/anti-CD28.

MSCs reduced IFN-γ, IL-17, and IL-9 cytokine production

The proportion of IL-9-, IL-17-, and IFN-γ producing cells was determined following intracellular staining in naive and non-naive isolated cells from patients and HD after treatment in the presence or absence of MSCs.

Table 4 Phenotyping of non-naive $CD4^+$ T cells from healthy donors (HD) and patients (Pat) with/without MSCs

	HD		Pat	
	MSCs	**No MSCs**	**MSCs**	**No MSCs**
TEMRA $CD45RA^+CD27^-$				
% Absolute count	2.0 ± 1.8 (1.4; 0.6–4.6) 600 ± 540 (420; 180–1380)	2.7 ± 1.5 (2.8; 0.9–4.4) 810 ± 450 (840; 270–1320)	4.7 ± 5.2 (2.7; 1.0–12.2) 1410 ± 1560 (810; 300–3660)	2.5 ± 1.1 (2.3; 1.5–4.0) 750 ± 330 (690; 450–1200)
Naive $CD45RA^+CD27^+$				
% Absolute count	6.5 ± 2.4 (6.1; 4.2–9.6) 1950 ± 720 (1830; 1260–2880)	9.5 ± 7.5 (8.3; 2.6–19.0) 2850 ± 2250 (2850; 780–5700)	14.3 ± 16.2 (8.3; 2.5–38.2) 4290 ± 4860 (2490; 750–11,460)	6.6 ± 4.8 (7.1; 1.4–11.0) 1980 ± 1440 (2130; 420–3300)
Memory $CD45RA^+CD27^+$				
% Absolute count	64.4 ± 29.5 (77.4; 20.3–82.6) 19,320 ± 8850 (23,220; 6090–24,780)	35.5 ± 15.9 (40.0; 12.6–49.5) 10,650 ± 4770 (12,000; 3780–14,850)	49.6 ± 28.9 (52.1; 16.3–77.9) 14,880 ± 8670 (14,850; 4890–23,370)	37.1 ± 21.9 (43.3; 6.8–54.9) 11,130 ± 6570 (12,990; 2040–16,470)
Effector $CD45RA^-CD27^-$				
% Absolute count	27.1 ± 29.3 (13.1; 11.4–71.0) 8130 ± 8790 (3930; 3420–21,300)	52.9 ± 20.6 (46.8; 36.4–81.6) 15,870 ± 6180 (14,040; 10,920–24,480)	31.4 ± 30.6 (17.5; 13.6–77.1) 9420 ± 9180 (5250; 4080–23,130)	53.7 ± 25.4 (47.4; 32.4 – 87.8) 16,110 ± 7620 (16,110; 9720–26,340)

Phenotype switch of isolated non-naive $CD4^+$ T-cells from healthy donors (HD) and patients (Pat) in presence/absence of MSCs after 6 days of Th17 stimulation. Average percentages and absolute count of the different phenotypes of all $CD4^+$ T-cells are shown; Values are shown as follows: mean ± standard deviation (median; minimum–maximum)

There were no significant differences between the four phenotypes in Pat or HD with MSCs vs. without MSCs (Mann–Whitney-U-Test)

Figure 3 show representative flow cytometric plots for the cytokine production of naive (Fig. 3A–D) and non-naive (Fig. 3E–H) CD4 + T-cells of Patient 1 with RA (Table 1) after Th17 stimulation in presence and absence of MSCs. Both, patients with inflammatory arthritis and HD showed almost similar patterns of flow cytometric plots, albeit with different percentages of cytokine production.

In general, non-naive isolated cells showed higher proportions of cytokine-producing cells following stimulation by Th17 polarizing conditions. Memory ($CD45RA^-CD27^+$) and especially effector ($CD45RA^-CD27^-$) $CD4^+$ T-cells produced high percentages of cytokine in the absence of MSCs. However, in the presence of MSCs the cytokine production was impaired (Table 6). The proportion of IL-9-, IL-17-, and IFN-γ-producing $CD4^+$ T-cells, especially in the memory and effector subpopulations, was reduced compare to the proportion of these cells in the absence of MSCs (Table 6). Although, the cytokine production in the previously naive fraction was lower compared to the counterpart (non-naive fraction), the same cytokine production retarding effect was observed in the presence of MSCs (Table 5). Cytokine contribution by naive ($CD45RA^+CD27^+$) and TEMRA ($CD45RA^+CD27^-$) cells appeared very low in both fractions. Nevertheless, a reduction in their contribution by IL-9, IL-17, and IFN-γ was determined in both but more pronounced in the non-naive fraction (Tables 5, 6). Comparing the influence of MSCs on cytokine production in patients and HD, it appears that IL-17 and IL-9, which are mainly produced by effector cells, are significantly reduced in patients by MSCs. Although not significant, this trend of reduced IL-9 (naive- and non-naive fraction: $p = 0.114$) and IL-17 (naive fraction $p = 0.114$ and non-naive fraction: $p = 0.486$) production in the presence of MSCs could also be observed in HD. In contrast, IFNγ production was significantly reduced in HD, mainly in the memory and naïve subpopulations (Fig. 2).

Discussion

The effect on the phenotype and cytokine production profile of naive and non-naive $CD4^+$ T-cells from patients with T-cell mediated autoimmune arthritis and HD under stimulation with Th17-polarizing conditions in the presence or absence of MSCs was determined. We could show, that co-culture of MSC with previously naïve or non-naïve $CD4^+$ T cells results in an abrogation of the differentiation into the effector T cell subtype and significantly reduced production of IFN-γ, IL-17 and IL-9 in both T-cell fractions derived from patients with autoimmune arthritis and from HD.

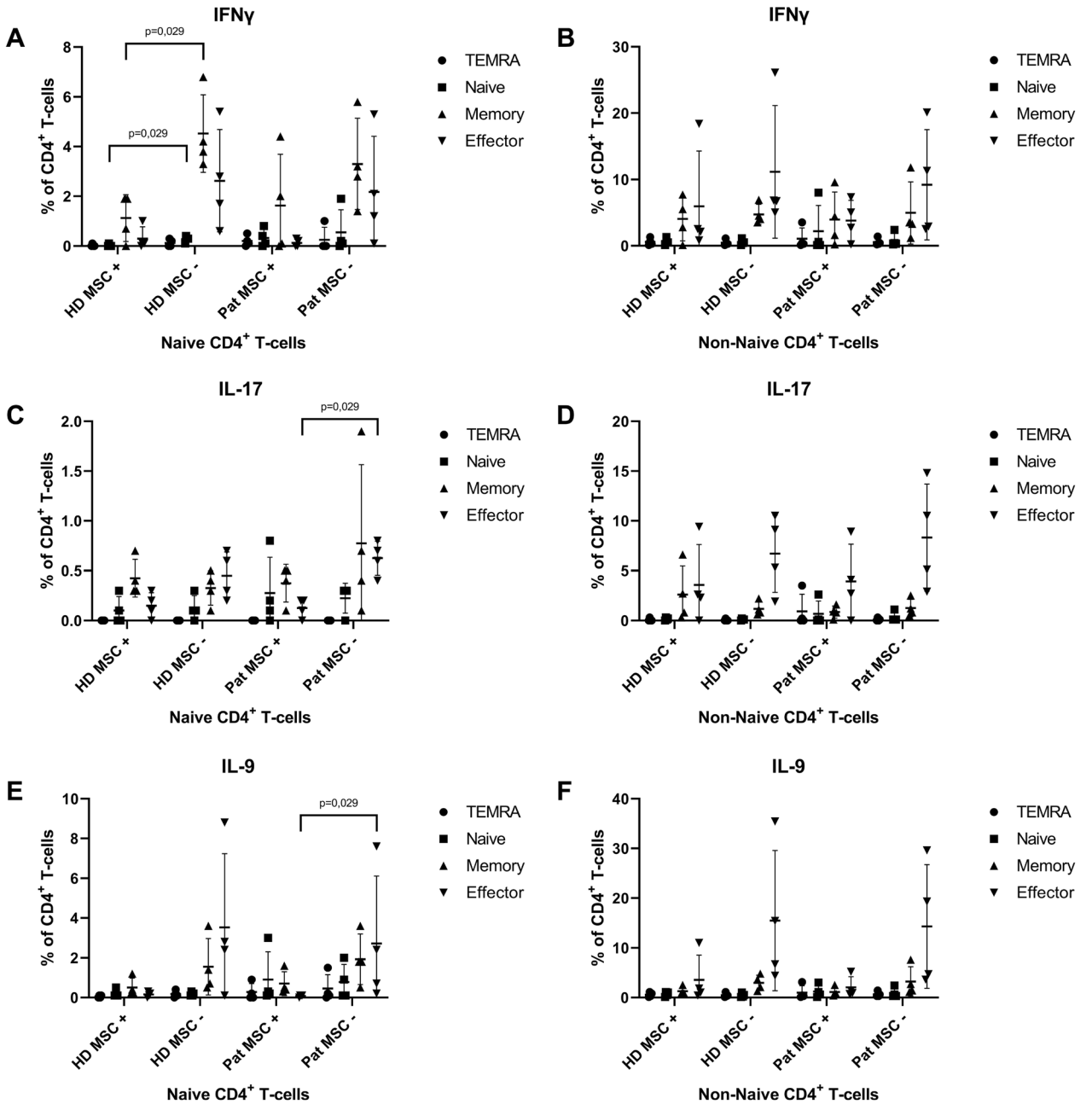

Fig. 2 Dot plots of IFNγ (**A** and **B**). IL-17 (**C** and **D**) and IL-9 (**E** and **F**) production of $CD4^+$ T-cells in the shown phenotypes (TEMRA, Naive, Memory and Effector) in patients and healthy donors. based on isolated naive and non-Naive $CD4^+$ T-cells after 6 days of Th17 stimulation. HD = healthy donors; Pat = patients; MSC − = without MSCs; MSC + = with MSCs; p-values are indicated (Mann–Whitney-U-Test)

The differentiation from naive ($CD45RA^+CD27^+$) and memory ($CD45RA^-CD27^+$) cells into effector ($CD45RA^-CD27^-$) or TEMRA ($CD45RA^+CD27^-$) cells was impaired in the presence of MSCs. Our findings indicate that MSCs prevent the differentiation of naive and memory cells under pro-inflammatory conditions. Preservation of the naive phenotype in co-culture with MSCs has already been shown in other studies [29, 39, 40]. Additionally, Batorov et al. showed that autologous MSCs can increase the recovery of lymphocytes in the naive stage after autologous hematopoietic stem cell transplantation in vivo and this process could be also observed in memory cells [41].

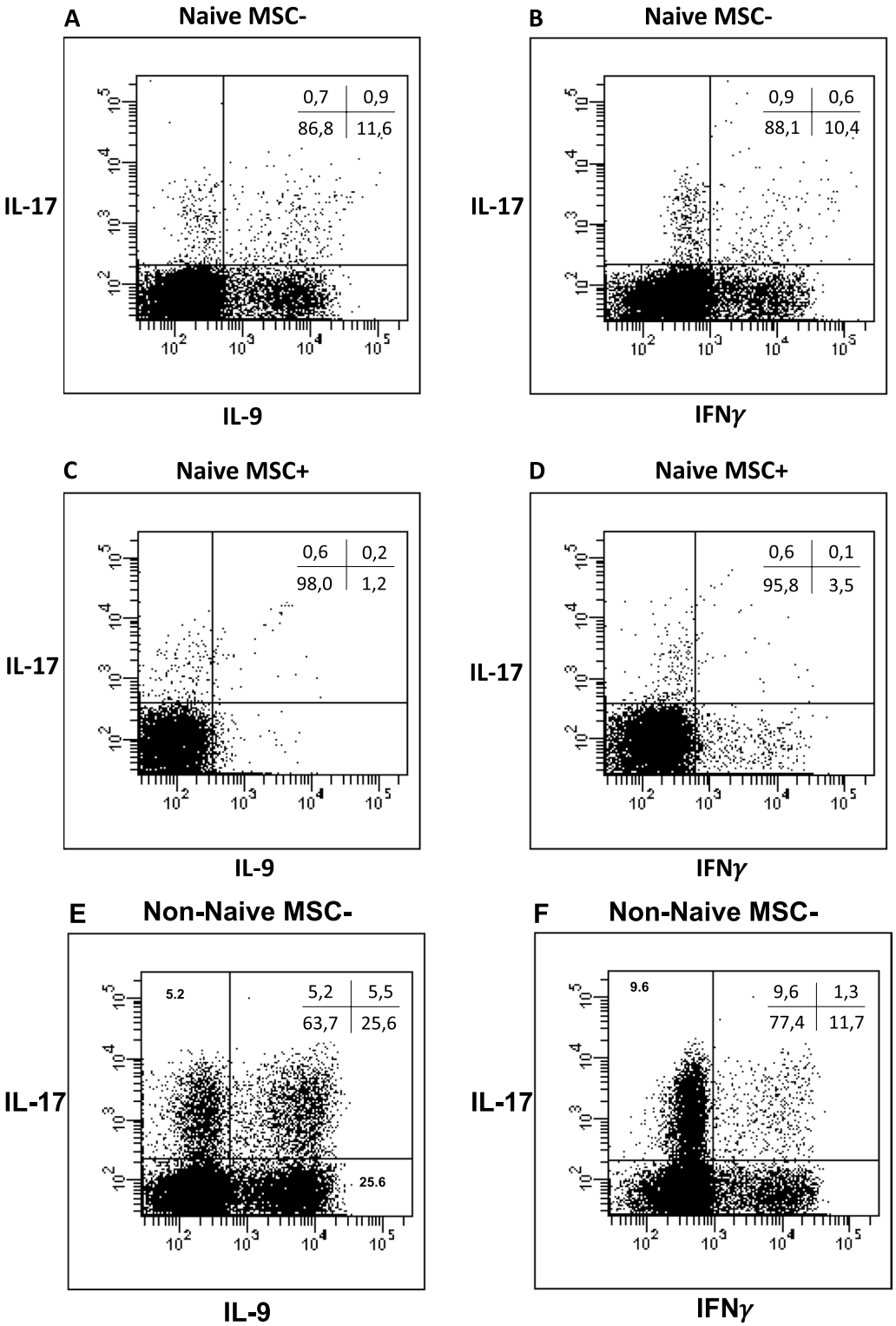

Fig. 3 Representative FACS plots for the cytokine production of naive (**A–D**) and non-naive (**E–H**) $CD4^+$ T-cells of Patient 1 with RA (Table 1) after Th17 stimulation. Data in the upper right area of each FACS plot indicate the percentage of $IL\text{-}17^+$, $IL\text{-}9^+$, $IL\text{-}17^+/IL\text{-}9^+$ and $IL\text{-}17^-/IL\text{-}9^-$ $CD4^+$ T-cells in the naive and non-naive fraction. **A** and **E** IL-17 and IL-9 production of naive/non-naive $CD4^+$ T-cells without MSCs (MSC–); **B** and **F** IL-17 and IFNγ production of naive/non-naive $CD4^+$ T-cells without MSCs (MSC–); **C** and **G** IL-17 and IL-9 production of naive/non-naive $CD4^+$ T-cells with MSCs (MSC+); **D** and **H** IL-17 and IFNγ production of naive/non-naive $CD4^+$ T-cells with MSCs (MSC+)

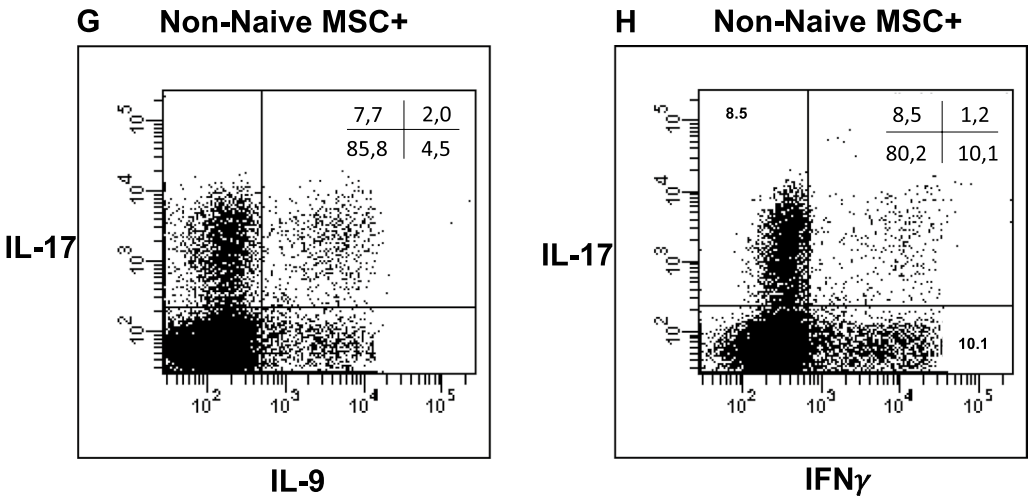

Fig. 3 continued

A possible mechanism of regulation of MSCs on T-cells may be by enhancing the proportion of regulatory T-cells. MSCs promote the induction of Treg cells in vivo and in vitro [42, 43]. Conversion of conventional T-cells into Treg is facilitated in the presence of MSCs [44, 45]. Liu et al. showed that MSCs could enhance $CD8^{+}CD28^{-}$ Treg cell activity by inhibiting naive $CD4^{+}$ T-cell activation and decreasing IFN-γ production in activated $CD4^{+}$ T-cells [46]. Consistent with the results of our experiments, intracellular IFN-γ production was significantly higher in "effector"-sorted cells from HD which were not co-cultered with MSC compared to MSC + cultures. Although no significant difference could be observed, the reduction in IFN-γ production in the presence of MSCs could also be shown in patients (Fig. 2). This might rise the impression that the immunomodulatory effect of MSCs is mitigated in patients. In addition, patients produced less cytokines than HD after stimulation. Reduced IFN-γ levels in the patient group might be explained by the substantial individual variations in the cytokine-mediated immunological microenvironment at different stages of disease development and medication. Additionally, and in contrast to the suspected unilateral IFN-γ suppression by MSCs, recent studies indicate that IFN-γ is a key factor in the management of RA patients with MSCs [47–49]. He et al. showed that the combination of IFN-γ and MSC can increase the clinical effectiveness of MSC-based therapy. Hypothetically, therefore, it would not make sense to significantly decrease IFN-γ production by MSCs in patients with rheumatic diseases which is also reflected in the results of our study.

Present results demonstrate the fundamental role of IL-31/IL-33 axis in the development of chronic inflammatory immune-mediated diseases via stimulating pro-inflammatory cytokines, regulating cell proliferation and controlling tissue remodeling. In addition, it is assumed that inhibition of IL-33 increases the expansion of Tregs and myeloid-derived suppressor cells and thus causes inhibition of Th17 cells. Although the direct influence of MSCs on the IL-31/IL-33 axis was not examined in our study, there is evidence that IL-33 can modulate MSCs and thus most likely targeting Th1/Th17 axis [50]. There is increasing evidence that IL-17 plays a major role in various steps in the development of RA, SLE and other autoimmune and chronic inflammatory diseases. Furthermore IL-17 is important for the elimination of extracellular and intracellular pathogens [51–53].

High levels of IL-17 are accompanied by an increase in the number of Th17 cells. These cells are associated with dysfunction of Treg cells observed in diseases such as RA, systemic sclerosis (SSc), and inflammatory bowel diseases (IBD) and support osteoclast stimulation by osteoblast- and osteocyte derived RANKL thereby promoting bone loss [54–58]. Our in vitro model demonstrated that the presence of MSCs reduced the number of IL-17 producing $CD4^{+}$ T-cells in patients and in HD despite being in a Th17 polarizing microenvironment. So far unknown secondary changes in gene regulation in MSC exerted by such pro-inflammatory environments may also modulate this effect. It has been described in earlier work that TLR4 stimulation can trigger proinflammatory cascades in MSC, which comprise secretion of besides others IL1b, IL6, IL8 and finally wnt5A and ROR2 expression as first response mechanisms after injury and in the very early phase of osteogenic differentiation. The latter may be again relevant in the crosstalk between MSC and T-cells as non-canonical wnt-signaling has immunomodulatory properties [59, 60]. MSCs also promote polarization of

Table 5 Cytokine production of naive CD4$^+$ T-cells from healthy donors (HD) and patients (Pat) with/without MSCs

	HD		Pat	
	MSCs	**No MSCs**	**MSCs**	**No MSCs**
Cytokine production % CD4$^+$ T-cells Absolute count				
IFNγ-production				
TEMRA in % Absolute count	0.0 ± 0.1 (0.0; 0.0–0.1) 0 ± 30 (0; 0–30)	0.1 ± 0.2 (0.1; 0.0–0.3) 30 ± 60 (30; 0–90)	0.2 ± 0.2 (0.2; 0.0–0.5) 60 ± 60 (60; 0–150)	0.3 ± 0.5; (0.0; 0.0–1.0) 90 ± 150 (0; 0–300)
Naive in % Absolute count	0.0 ± 0.1 (0.0; 0.0–0.1) 0 ± 30 (0; 0–30)	0.3 ± 0.1 (0.3; 0.1–0.4)[a] 90 ± 30 (90; 30–120)	0.3 ± 0.4 (0.3; 0.0–0.8) 90 ± 120 (90; 0–240)	0.6 ± 0.9 (0.2; 0.0–1.9) 180 ± 270 (60; 0–570)
Memory in % Absolute count	1.1 ± 0.9 (1.3; 0.0–1.9) 330 ± 270 (390; 0–570)	4.5 ± 1.6 (4.0; 3.3–6.8)[b] 1350 ± 480 (1200; 990–2040)	1.6 ± 2.1 (1.1; 0.0–4.4) 480 ± 630 (330; 0–1320)	3.3 ± 1.8 (3.0; 1.4–5.8) 990 ± 540 (900; 420–1740)
Effector in % Absolute count	0.3 ± 0.5 (0.1; 0.0–0.1) 90 ± 150 (30; 0–30)	2.6 ± 2.1 (2.3; 0.6–5.4) 780 ± 630 (690; 180–1620)	0.1 ± 0.2 (0.1; 0.0–0.3) 30 ± 60 (30; 0–90)	2.2 ± 2.2 (1.7; 0.1–5.3) 660 ± 660 (510; 30–1590)
IL-17-production				
TEMRA in % Absolute count	0.0 ± 0.0 (0.0; 0.0–0.0) 0 ± 0 (0; 0–0)	0.0 ± 0.0 (0.0; 0.0–0.0) 0 ± 0 (0; 0–0)	0.0 ± 0.0 (0.0; 0.0–0.0) 0 ± 0 (0; 0–0)	0.0 ± 0.0 (0.0; 0.0–0.0) 0 ± 0 (0; 0–0)
Naive in % Absolute count	0.1 ± 0.1 (0.1; 0.0–0.3) 30 ± 30 (30; 0–90)	0.1 ± 0.1 (0.1; 0.0–0.3) 30 ± 30 (30; 0–90)	0.3 ± 0.4 (0.2; 0.0–0.8) 90 ± 120 (60; 0–240)	0.2 ± 0.2 (0.3; 0.0–0.3) 60 ± 60 (90; 0–90)
Memory in % Absolute count	0.4 ± 0.2 (0.4; 0.3–0.7) 120 ± 60 (120; 90–210)	0.3 ± 0.2 (0.4; 0.1–0.7) 90 ± 60 (120; 30–210)	0.4 ± 0.2 (0.5; 0.1–0.5) 120 ± 60 (150; 30–150)	0.8 ± 0.8 (0.6; 0.1–1.9) 240 ± 240 (180; 30–570)
Effector in % Absolute count	0.2 ± 0.1 (0.2; 0.0–0.3) 60 ± 30 (60; 0–90)	0.5 ± 0.2 (0.5; 0.2–0.7) 150 ± 60 (150; 60–210)	0.1 ± 0.1 (0.2; 0.0–0.2) 30 ± 30 (60; 0–60)	0.6 ± 0.2 (0.7; 0.4–0.8)[c] 180 ± 60 (210; 120–240)
IL-9-production				
TEMRA in % Absolute count	0.1 ± 0.1 (0.1; 0.0–0.1) 30 ± 30 (30; 0–30)	0.2 ± 0.2 (0.2; 0.0–0.4) 60 ± 60 (60; 0–120)	0.3 ± 0.4 (0.1; 0.0–0.9) 90 ± 120 (30; 0–270)	0.5 ± 0.7 (0.2; 0.0–1.5) 150 ± 210 (60; 0–450)
Naive in % Absolute count	0.2 ± 0.2 (0.2; 0.1–0.5) 60 ± 60 (60; 30–150)	0.2 ± 0.1 (0.2; 0.1–0.3) 60 ± 30 (60; 30–90)	0.9 ± 1.4 (0.3; 0.1–3.0) 270 ± 420 (90; 30–900)	0.8 ± 0.9 (0.5; 0.1–2.0) 240 ± 270 (150; 30–600)
Memory in % Absolute count	0.5 ± 0.5 (0.3; 0.2–1.2) 150 ± 150 (90; 60–360)	1.6 ± 1.4 (1.1; 0.5–3.6) 480 ± 420 (330; 150–1080)	0.7 ± 0.6 (0.5; 0.3–1.6) 210 ± 180 (150; 90–480)	1.9 ± 1.3 (1.8; 0.5–3.6) 570 ± 390 (480; 150–1080)
Effector in % Absolute count	0.2 ± 0.1 (0.2; 0.0–0.3) 60 ± 30 (60; 0–90)	3.5 ± 3.7 (2.6; 0.1–8.8) 1050 ± 1110 (780; 30–2640)	0.1 ± 0.1 (0.1; 0.0–0.1) 30 ± 30 (30; 0–30)	2.7 ± 3.4 (1.6; 0.2–7.6)[d] 810 ± 1020 (2640; 60–2280)

Average percentage of cytokine producing positive cells and absolute count within the CD4$^+$ T cell subpopulations of TEMRA, Naive, Memory and Effector cells based on naive CD4$^+$ T-cells after Th17 stimulation over 6 days. HD = healthy donors. Pat = patients. Values are shown as follows: mean ± standard deviation (median; minimum–maximum)

[a] Differences between IFNγ-production of the naive phenotype in HD with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

[b] Differences between IFNγ-production of the memory phenotype in HD with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

[c] Differences between IL-17-production of the effector phenotype in Pat with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

[d] Differences between IL-9-production of the effector phenotype in Pat with MSCs vs. without MSCs: $p = 0.029$ (Mann–Whitney-U-Test)

There were no significant differences between the cytokine production of other phenotypes in Pat or HD with MSCs vs. without MSCs (Mann–Whitney-U-Test)

monocytes/macrophages into anti-inflammatory type 2 phenotype increasing levels of IL-10 having as consequence reduction in the levels of TNFa and IL-17 [61, 62] and is in accordance with our findings.

Interestingly, naive and non-naive CD4$^+$ T-cells stimulated under Th17 conditions in our system induce production of high levels of IL-9. IL-9 is a cytokine produced by various cells including Th2, Th17, Treg, and Th9 cells

Table 6 Cytokine production of non-naive CD4$^+$ T-cells from healthy donors (HD) and patients (Pat) with/without MSCs

	HD		Pat	
	MSCs	**No MSCs**	**MSCs**	**No MSCs**
Cytokine production % CD4$^+$ T-cells Absolute count				
IFNγ-production				
TEMRA in % Absolute count	0.6 ± 0.5 (0.5; 0.1–1.3) 180 ± 150 (150; 30–390)	0.5 ± 0.4 (0.3; 0.1–1.1) 150 ± 120 (90; 30–90)	1.1 ± 1.6 (0.4; 0.1–3.5) 330 ± 480 (180; 30–1050)	0.6 ± 0.6; (0.4; 0.2–1.4) 180 ± 180 (120; 60–420)
Naive in % Absolute count	0.7 ± 0.5 (0.7; 0.1–1.3) 210 ± 150 (210; 30–390)	0.5 ± 0.4 (0.5; 0.1–1.1) 150 ± 120 (150; 30–330)	2.2 ± 3.9 (0.4; 0.1–8.0) 660 ± 1170 (120; 30–2400)	0.8 ± 1.1 (0.4; 0.2–2.4) 240 ± 330 (120; 60–720)
Memory in % Absolute count	4.0 ± 3.3 (4.2; 0.1–7.7) 1200 ± 990 (1260; 30–2310)	4.7 ± 1.5 (4.3; 3.5–6.9) 1410 ± 450 (1290; 1050–2070)	4.0 ± 4.2 (3.0; 0.2–9.6) 1200 ± 1260 (900; 60–2880)	5.0 ± 4.7 (3.4; 1.2–11.8) 1500 ± 1410 (1020; 360–3540)
Effector in % Absolute count	6.0 ± 8.3 (2.3; 0.8–18.4) 1800 ± 2490 (690; 240–5520)	11.2 ± 10.0 (6.8; 5.0–26.1) 3360 ± 3000 (2040; 1500–7830)	3.8 ± 3.0 (3.9; 0.2–7.3) 1140 ± 900 (1170; 60–2190)	9.2 ± 8.3 (7.1; 2.5–20.1) 2760 ± 2490 (2130; 750–6030)
IL-17-production				
TEMRA In % Absolute count	0.1 ± 0.2 (0.0; 0.0–0.3) 30 ± 60 (0; 0–90)	0.1 ± 0.1 (0.0; 0.0–0.2) 30 ± 30 (0; 0–60)	0.9 ± 1.7 (0.1; 0.0–3.5) 270 ± 510 (30; 0–1050)	0.1 ± 0.1 (0.1; 0.0–0.3) 30 ± 30 (30; 0–90)
Naive in % Absolute count	0.2 ± 0.1 (0.2; 0.0–0.3) 60 ± 30 (60; 0–90)	0.1 ± 0.1 (0.1; 0.0–0.2) 30 ± 30 (30; 0–60)	0.7 ± 1.3 (0.1; 0.0–0.1) 210 ± 390 (30; 0–30)	0.4 ± 0.5 (0.1; 0.1–1.1) 120 ± 150 (30; 30–330)
Memory in % Absolute count	2.6 ± 2.9 (1.8; 0.3–6.6) 780 ± 870 (540; 90–1980)	1.2 ± 0.7 (1.0; 0.6–2.2) 360 ± 210 (300; 180–660)	0.9 ± 0.6 (0.9; 0.1–1.6) 270 ± 180 (270; 30–480)	1.3 ± 0.9 (1.0; 0.5–2.5) 390 ± 270 (300; 150–750)
Effector in % Absolute count	3.6 ± 4.1 (2.5; 0.0–9.4) 1080 ± 1230 (750; 0–2820)	6.7 ± 3.9 (7.2; 1.9–10.5) 2010 ± 1170 (2160; 570–3150)	3.9 ± 3.7 (3.4; 0.0–8.9) 1170 ± 1110 (1020; 0–2670)	8.3 ± 5.4 (7.8; 2.9–14.8) 2490 ± 1620 (2340; 870–4440)
IL-9-production				
TEMRA in % Absolute count	0.6 ± 0.5 (0.6; 0.1–1.1) 180 ± 150 (180; 30–330)	0.5 ± 0.4 (0.4; 0.1–1.1) 150 ± 120 (120; 30–330)	1.0 ± 1.4 (0.4; 0.1–3.1) 300 ± 420 (120; 30–930)	0.7 ± 0.5 (0.5; 0.3–1.4) 210 ± 150 (150; 90–420)
Naive in % Absolute count	0.7 ± 0.5 (0.8; 0.1–1.1) 210 ± 150 (240; 30–330)	0.5 ± 0.4 (0.4; 0.1–1.0) 150 ± 120 (120; 30–300)	1.2 ± 1.3 (0.9; 0.1–3.0) 360 ± 390 (270; 30–900)	0.9 ± 1.0 (0.5; 0.3–2.4) 270 ± 300 (150; 90–720)
Memory in % Absolute count	1.3 ± 0.8 (0.9; 0.8–2.5) 390 ± 240 (270; 240–750)	3.0 ± 1.6 (2.9; 1.3–4.8) 900 ± 480 (870; 390–1440)	1.1 ± 0.9 (0.8; 0.4–2.5) 330 ± 270 (240; 120–750)	3.2 ± 3.0 (2.1; 1.1–7.6) 960 ± 900 (630; 330–2280)
Effector in % Absolute count	3.6 ± 5.0 (1.4; 0.4–11.0) 1080 ± 1500 (420; 120–3300)	15.5 ± 14.1 (11.1; 4.4–35.4) 4650 ± 4230 (3330; 1320–10,620)	2.1 ± 2.1 (1.2; 0.7–5.2) 630 ± 630 (360; 210–1560)	14.3 ± 12.5 (12.0; 3.6–29.6) 4290 ± 3750 (3600; 1080–8880)

Average percentage of cytokine producing positive cells and absolute count within the CD4$^+$ T-cell subpopulations of TEMRA, Naive, Memory and Effector cells based on non-naive CD4$^+$ T-cells after Th17 stimulation over 6 days. HD = healthy donors. Pat = patients. Values are shown as follows: mean ± standard deviation (median; minimum–maximum); There were no significant differences between the cytokine production of the phenotypes in Pat or HD with MSCs versus without MSCs (Mann–Whitney-U-Test)

and can be mainly produced by stimulation in the presence of IL-4 and TGFβ [8, 63]. IL-9 is important in the regulation of the airway inflammation inducing proliferation of goblet cells and mucin producing cells [64]. However, IL-9 has been related in some pathogenic processes of diseases such as lupus erythematosus, RA, and SSc [28, 65–67]. Our results are in line with a recently published article of our group, showing the Th17-inducing cytokines are effective to induce IL-9 production and Th9-polarized cells [33].

This study has several limitations. First, we included a small number of patients associated with different underlying rheumatic diseases, pathophysiological aspects, and therapeutical characteristics comprising different doses of corticosteroids and immunosuppressants. Due to small numbers, outliers may prominently contribute

to the differences in results. Although our study is limited by the small case numbers, the highly explorative character of the experiments allow generation of the hypothesis that MSC are able to reduce the differentiation into cytokine-producing effector helper T cells and clearly showed reduction of pro-inflammatory cytokines, such as IL-17, IFN-γ and IL-9. The use of the two magnetic-bead-based isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) for the cell isolation of naive and non-naive $CD4^+$ cells with an average purity of >90% leads to the assumption that the remaining 10% of the cell fractions might be contaminating cell subpopulations, which could falsely contribute to the investigated subpopulations and possibly to overall cytokine production after expansion in vitro. It should therefore be mentioned that the data gained from the non-naïve $CD4^+$ T cell fraction may include cytokine-producing cells of the complementary fraction.

Applying a transwell system, we could prove that these effects were exclusively based on soluble factors and not on direct cell–cell-contacts between MSCs and sorted naïve or non-naïve T cells, respectively. Although we failed to measure soluble factors in the supernatants due to technical limitations, as we had to remove the fairly small amounts T cells by several centrifugation steps, our study showed that the presence of MSCs reduced the proportion of IL-9, IL-17, and IFN-γ producing cells, suggesting a modulatory effect of the MSCs on T-cells inhibiting the differentiation of naive and memory cells into cytokine producing effector T-cells even in pro-inflammatory conditions. In addition, however, it should be mentioned that the expression of individual cytokines by Th1, Th9 and Th17 for each Th lineage may ultimately be biased, as no other driving cytokines and their effect on Th cells, such as IL-12 on Th1 or IL-23 on Th17, have been addressed. Of note, intracellular cytokine production does not automatically mean cytokine release and provides a indirectly view on cytokine producing ability of defined Th subpopulations.

Conclusion

The distribution of different T-cell phenotypes in the course of autoimmune diseases seems to play an important role for targeted therapeutic approaches with the aim of reducing the proinflammatory cytokine production in order to control the immune response [68]. Today, various known inhibitors like monoclonal antibodies against TNF-α (e.g. Adalimumab) and IL-6 (Tocilizumab) receptor, TNF-α soluble receptor, IL-1 receptor antagonist and analogous tools are available, targeting the IL-17 pathway [53].

As a cell-based therapy, MSCs might represent a promising alternative for patients who do not respond to a conventional therapy with disease-modifying antirheumatic drugs or biologicals [29]. Anyway, preventing the phenomenon of T-cell differentiation into effector cells or using MSCs as a prophylactic option in affected patients should be addressed in pre-clinical and clinical studies.

Acknowledgements
The authors would like to thank the research group headed by Professor Norbert Schütze, Orthopedic Clinic, König-Ludwig-Haus, Würzburg, Germany for providing the mesenchymal stem cells.

Author contributions
MR and MP wrote the main manuscript. All authors reviewed, read and approved the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. We acknowledge support for the Article Processing Charge from the DFG (German Research Foundation, 491454339).

Availability of data and materials
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval was obtained from the local ethics committee of the Medical Faculty of the University Hospital of Würzburg (No. 123/14). Informed consent was obtained from all subjects and/or their legal guardian(s). All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by a named institutional and/or licensing committee.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 8 December 2022 Accepted: 19 July 2023
Published online: 31 July 2023

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