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Pathogenic Transdifferentiation of Th17 Cells Contribute to Perpetuation of Rheumatoid Arthritis during Anti-TNF Treatment

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T-helper cells producing interleukin (IL)-17A and IL-17F cytokines (Th17 cells) are considered the source of autoimmunity in rheumatoid arthritis (RA). In this study, we characterized specific pathogenic features of Th17 cells in RA. By using nano-string technology, we analyzed transcription of 419 genes in the peripheral blood CCR6$^+$CXCR3$^-$ CD4$^+$ cells of 14 RA patients and 6 healthy controls and identified 109 genes discriminating Th17 cells of RA patients from the controls. Th17 cells of RA patients had an aggressive pathogenic profile and in addition to signature cytokines *IL-17*, *IL-23* and *IL-21*, and transcriptional regulators RAR-related orphan receptor gamma of T cells (*RORγt*) and Janus kinase 2 (*JAK2*), they produced high levels of *IL-23R*, C-C chemokine ligand type 20 (*CCL20*), granulocyte-monocyte colony-stimulating factor (*GM-CSF*) and transcription factor *Tbet* required for synovial homing. We showed that Th17 cells are enriched with Helios-producing Foxp3- and IL2RA-deficient cells, indicating altered regulatory profile. The follicular T-helper (Tfh) cells presented a functional profile of adaptor molecules, transcriptional regulator *Bcl-6* and B-cell activating cytokines *IL-21*, *IL-31* and leukemia inhibitory factor (*LIF*). We observed that anti–tumor necrosis factor (TNF) treatment had a limited effect on the transcription signature of Th17 cells. Patients in remission retained the machinery of receptors (*IL-23R* and *IL-1R1*), proinflammatory cytokines (*IL-17F*, *IL-23*, *IL-21* and *TNF*) and adaptor molecules (C-X-C chemokine receptor 5 (*CXCR5*) and cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*)), essential for efficient transdifferentiation and accumulation of Th17 cells. This study convincingly shows that the peripheral blood CCR6$^+$CXCR3$^-$ CD4$^+$ cells of RA patients harbor pathogenic subsets of Th17 and Tfh cells, which may transdifferentiate from Tregs and contribute to perpetuation of the disease.
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INTRODUCTION

The Th17 cells, a subpopulation of T-helper cells characterized by high production of the cytokines interleukin (IL)-17A and IL-17F, are associated with the induction of autoimmunity and have important roles in the pathogenesis of rheumatoid arthritis (RA) (1). In the rheumatoid synovium, Th17 cells are enriched and produce bioactive IL-17 in response to stimulation. The produced IL-17 amplifies the inflammation induced by other cytokines, particularly tumor necrosis factor (TNF) and IL-1 (2,3), and induces extensive inflammatory cell migration and massive cartilage and bone degradation (4). The prevalence of IL-17 producing CD4 T-cells is increased in the circulation of RA patients (5) and correlates to the number of swollen joints and to systemic inflammation (6). Importantly, a reduction of circulating Th17 cells has been a suggested prerequisite of clinically efficient treatment in RA (7–9). IL-17 was targeted in several clinical studies for RA (10). Currently, clinical trials with monoclonal antibodies against IL-17 and IL-17 receptor report a favorable therapeutic effect in patients with severe arthritis (11–13).

It has been postulated and experimentally proven that Th17 cells may differentiate to functionally distinct cells depending on cytokines directing their development (14). An inflammatory envi-

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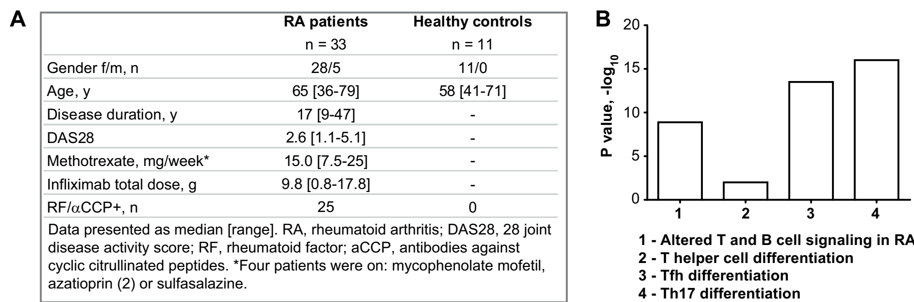

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	RA patients	Healthy controls
	n = 33	n = 11
Gender f/m, n	28/5	11/0
Age, y	65 [36-79]	58 [41-71]
Disease duration, y	17 [9-47]	-
DAS28	2.6 [1.1-5.1]	-
Methotrexate, mg/week*	15.0 [7.5-25]	-
Infliximab total dose, g	9.8 [0.8-17.8]	-
RF/αCCP+, n	25	0

Data presented as median [range]. RA, rheumatoid arthritis; DAS28, 28 joint disease activity score; RF, rheumatoid factor; aCCP, antibodies against cyclic citrullinated peptides. *Four patients were on: mycophenolate mofetil, azatioprin (2) or sulfasalazine.

Figure 1. (A) Clinical characteristics of patients and controls included in the study. (B) Network analysis of differentially expressed genes in PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients and healthy controls using the Ingenuity IPA 5.5.1 program. Genes included the following: 1: *TLR10, TLR5, CXCL13, IL2, TLR6, TLR7, FASLG*; 2: *IL-2, CXCR5*; 3: *IL-21, IL-21R, ICOS, Stat3, CXCR5, IL-6, Bcl-6*; 4: *TGFBR1, IL-23R, TGFB1, IL-6, TBX21, Rorc, IL-17F, TNF, IL-17A*.

ronment in RA abundant with IL-1β and IL-6 is associated with maturation of Th17 cells from naive and Foxp3$^+$ regulatory T cells (15) (Tregs) and is characterized by surface expression of C-C chemokine receptor type 6 (CCR6), the induction of master transcription factor RAR-related orphan receptor gamma of T cells (RORγt) and production of signature cytokines IL-17A/IL-17F. Several transcription phases define development of Th17 (16), where production of IL-21 is characteristic of mature Th17 cells, acting as an amplification factor inducing expansion of Th17 cells and the expression of IL-23R. The expression of IL-23R has strong linkage to inflammation and has been shown to be critical for the pathogenic phenotype of Th17 cells (17,18). It is often accompanied by high production of the proinflammatory cytokines IL-22, TNF and granulocyte-monocyte colony-stimulating factor (GM-CSF). In healthy subjects, differentiation of Th17 cells is often controlled by combination of IL-6 and transforming growth factor (TGF)-β, and the balance with proinflammatory signaling may reprogram them to the Treg phenotype (19).

CCR6 appears to be the dominant receptor for the migration of Th17 cells to secondary lymphoid organs (20). The expression of CCR6 has been shown on autoreactive memory T cells (21). CCR6-expressing Th17 cells are preferentially recruited to the inflamed joints of RA patients attracted by large amounts of C-C chemokine ligand type 20 (CCL20) produced by rheumatoid T cells and synoviocytes (22). It was recently demonstrated that synovial fibroblasts assist transdifferentiation of Tregs into Th17 cells with a potent osteoclastogenic phenotype (23). In the present study, we performed transcriptional and functional analysis of circulating CCR6$^+$CXCR3$^-$ CD4$^+$ cells in RA patients on anti-TNF treatment and in healthy controls. We show that these cells displayed an evident pathogenic transcriptional profile characterized by high *IL-23R*, *CCL20*, *GM-CSF* and the combined upregulation of master transcription factors *RORγt* and *Tbet*. The enrichment of *Th17* cells may occur through transdifferentiation of Tregs and may harbor a strong triggering potential for the aberrant autoimmune processes in patients with active RA and in clinical remission.

MATERIALS AND METHODS

Patients

Blood samples were obtained from 33 patients (28 women, 5 men) with established RA. Clinical characteristics of the patients are presented in Figure 1A. The patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (24) and had a duration of disease of 17 years (range 9–47 years). All RA patients obtained regular treatment with monoclonal anti-TNF antibodies (infliximab, 3–5 mg/kg every 8 wks). A total of 29 patients were also treated with methotrexate (median dose 15 mg/week), 2 patients were treated with azathioprine (150 mg/day), 1 patient with salazopyrin and 1 patient with mycophenolate mofetil (2 g/day). Clinical activity of RA was calculated at the time of blood sampling based on the number of swollen and tender joints, erythrocyte sedimentation rate (ESR) and global health assessment of the patient, and the disease activity score (DAS28) was constructed (25). The patients with DAS28 <3.0 were considered in clinical remission. The control group was comprised of 11 healthy subjects, all women, median age 58 years (range 41–71 years). None of the controls reported any autoimmune disease or the use of any pharmacological drugs. For the gene transcription analysis, 14 of the RA patients were included. Exclusion criteria were (a) insufficient mRNA amount of isolated CCR6$^+$CXCR3$^-$ cells, (b) male gender and (c) antirheumatic treatment other than methotrexate. The study was approved by the Regional Ethics Board in Gothenburg, Sweden (Dnr 633-07).

Cell Isolation and Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by density gradient by using Lymphoprep (Axis-Shield PoC As). A total of 50 μL PBMCs was used for flow cytometry, and the remaining amount was subjected to selection of CCR6$^+$CXCR3$^-$ CD4$^+$ cells by using a cocktail of antibody-coated magnetic beads (human Th17 enrichment kit, #18162; STEMCELL Technologies). The isolated cells consisted of 85% CCR6$^+$CXCR3$^-$ cells and had a viability of 94%. CCR6$^+$CXCR3$^-$ CD4$^+$ cells were incubated in 10% fetal calf serum RPMI medium with and without phorbol 12-myristate 13-acetate (PMA, 30 ng/mL; Sigma-Aldrich), in combination with ionomycine (0.5 μg/mL; Sigma-Aldrich) for 4 h at 37°C, 5% CO^2. Supernatants were collected for cytokine analysis, and cell pellets were used for gene expression.

Flow Cytometry

The cells were prepared and stained for the fluorescence-activated cell sorter (FACS) analysis as previously described (26). Anti-CD4 (GK1.5), anti-CD27 (L128), and anti–C-X-C chemokine receptor 5 (anti-CXCR5; 2G8) were purchased from BD Biosciences; anti-CD19 (H1B19), anti-CD45RA (HI100), anti-CCR6 (G034E3), and anti-CXCR3 (G025H7) from BioLegend. Intracellular staining for Bcl-6 was performed as previously described (27), with anti-Bcl-6 (K112-91) and isotype control (mouse IgG1κ, MOPC-21), both from BD Biosciences. For blocking of unspecific binding via Fc receptors, polyclonal rabbit F(ab')2 anti-human immunoglobulin was added to cells before staining. Fluorochrome minus one (FMO) was used to determine negatively and positively stained populations when needed (28). Cells were collected, 5×10^5 events/sample, using a FACSCanto II (BD Bioscience) equipped with FACSDiva software. The analysis was performed using FlowJo software (version 10.0.6, Tree Star, Inc.). Compensation for fluorochrome interferences was done using single-stained CompBeads (BD Bioscience).

Gene Expression Analysis

RNA samples were prepared from cell lysates in Buffer RLT by using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The prepared RNA samples were analyzed with an nCounter Analysis System (NanoString Technologies) by using an HuTh17 CodeSet containing 419 genes. The obtained raw data were normalized with NanoString's nSolver™ analysis program by using the transcription of *β2-microglobulin*, ribosomal protein L3 (*RPL3*) and *β-actin* as housekeeping genes. A total of 387 genes, for which maximum expression level was above noise background, were selected for further analysis. The complete list of these genes is available on request.

Cytokine Production

Analysis of the cytokine production in the $CCR6^+CXCR3^-$ $CD4^+$ cultures was carried out by using Proteome Profiler™ Array, Human Cytokine Array Panel A (ARY005, RnD Systems), according to manufacturer's instructions. Equal amounts of culture medium were mixed with a cocktail of biotinylated detection antibodies. The mix was incubated with the array membrane to allow cytokine antibody complexes in the sample to bind to anti-cytokine antibodies captured on the membrane. After washing away unbound material, a streptavidin–horseradish peroxidase complex was added and detection of array spots was performed by using the Chemi Reagent Mix from the kit. Chemiluminescent signals were visualized and quantified by the Chemidoc equipment and Quantity-One software (Bio-Rad Laboratories). Pixel densities were normalized to reference spots on the membrane.

Statistical Analysis

The values are presented as median and range. The mean expression for each gene of 14 RA patients and 6 controls was calculated and compared by unpaired t test. The difference in groups was calculated as ratio. The ratio >1.5 and p value <0.05 indicated significant difference in gene expression. The pathway analysis was performed by using an Ingenuity IPA 5.5.1 program (Ingenuity) and publicly available databases.

RESULTS

Network Analysis

$CCR6^+CXCR3^-$ $CD4^+$ T cells of 14 RA patients and 6 healthy controls, all women, were polarized by PMA/ionomycin stimulation to determine the profile of the cells. The unsupervised clustering of 387 studied genes showed a clear separation pattern between the PMA/ionomycin-stimulated and nonstimulated cells. Among the stimulated cells, the RA patients were separated from the controls. Transcriptional analysis showed that 109 genes had significant (≥1.5-fold and $p < 0.05$) difference between the RA patients and healthy controls. The network analysis of differentially expressed genes was consistent with the presence of altered T-cell signaling, and Th17 and Tfh differentiation, characteristic for RA (Figure 1B).

$CCR6^+CXCR3^-$ $CD4^+$ T Cells of RA Patients Have a Pathogenic Th17 Gene Profile

The comparison of gene transcription in the PMA/ionomycin-stimulated $CCR6^+CXCR3^-$ $CD4^+$ cells of RA patients and healthy controls showed that RA patients had higher transcription of the signature cytokines *IL-17F*, *IL-17A*, and *IL-22*, which earlier was shown to characterize Th17-cells (29,30). These patients also had increased expression of the transcription factor RORγt (encoded by *Rorc*) controlling IL-17A/IL-17F production (Figure 2A). The $CCR6^+CXCR3^-$ $CD4^+$ cells of RA patients had features of memory cells, expressing high levels of CD62L (encoded by *SELL*) with well-established autocrine expansion of long-lived memory cells mediated by IL-2 and IL-24. Additionally, the $CCR6^+CXCR3^-$ $CD4^+$ cells of RA patients had all characteristic features associated with pathogenic properties of Th17 cells (17) and expressed the transcription factors Tbet (*Tbx21* gene), *Lef1* and *Egr-2*; the *CD226* receptor; and cytokines GM-CSF (*CSF2* gene) and *TNF* (Figure 2A). Cytokine profile array proved a significant enrichment of the cell supernatants with Th17 signature cytokines IL-17, GM-CSF (Figure 2B) and chemokine CCL5. The supernatants of RA patients contained high levels of TNF, IL-2 and PAI-1 compared with healthy controls, which verified proinflammatory and a readily proliferating phenotype of these cells. The production of cytokines characteristic for other Th subtypes (interferon [IFN]-γ, IL-13 [Figure 2B], IL-12p70 and MIF [not shown]) were on low levels and showed no differences for the supernatants of RA patients and controls. Notably, the mechanisms of Tbet upregulation in Th17 cells were independent of IFNγ, since intracellular media-

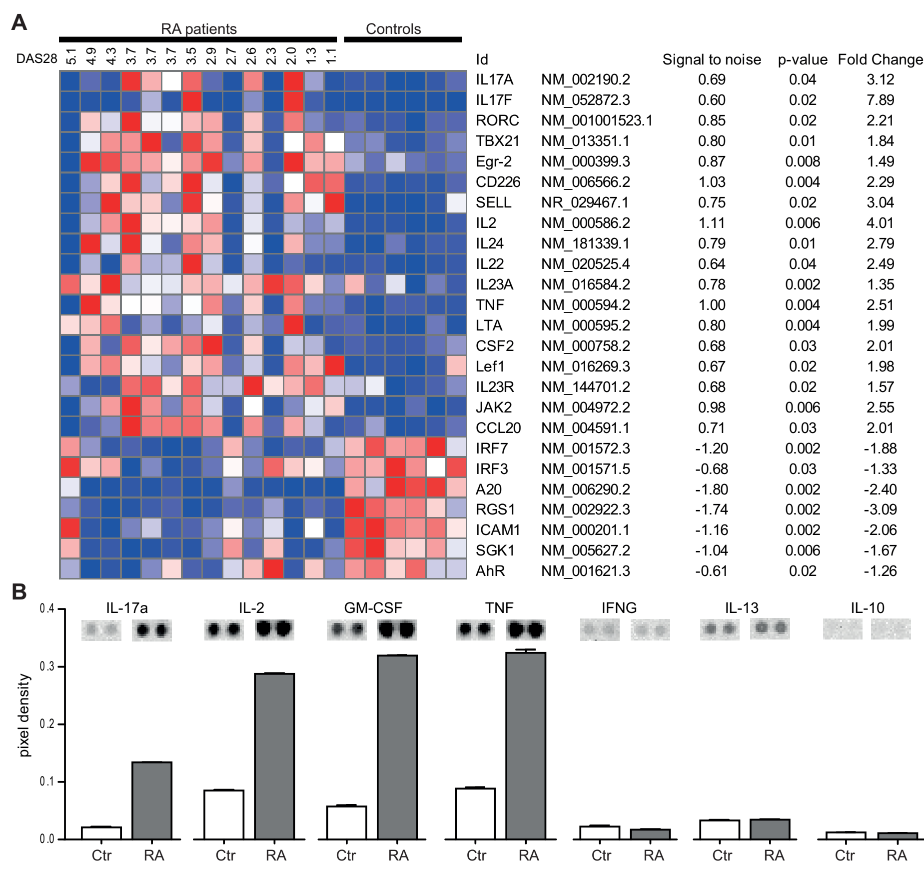

Figure 2. (A) Gene expression profiles of PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients and healthy controls. Shown are the differential expression levels for genes (rows) in patients or controls (columns). Normalized raw gene expression values from microarray experiments were used to identify differentially expressed genes involved in Th17 differentiation and function. DAS28, disease activity score based on 28 defined joints, is indicated for each RA patient. (B) Cytokines produced by PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients (n = 7) and healthy controls (n = 6). Equal amounts of cell culture medium were pooled and subjected to cytokine array analysis. Data shown in the bar graph are the results of densitometric analysis.

tors of IFNγ signaling (*IRF7* and *A20*) were repressed (Figure 2A).

The analysis of transcriptional profile of the effectors that polarize the differentiation of Th17 cells was carried out as reported (17). RA patients had increased production of *IL-23* with autocrine stimulation of *IL-23R* and activation of its intracellular mediator Janus kinase 2 (*JAK2*), enhancing further the expression of *CCL20* and proinflammatory cytokines *IL-22*, GM-CSF (*CSF2* gene), *TNF* and *lymphotoxin-α* (Figure 2A). This autocrine IL-23A stimulation could be traced both in the patients with high IL-17A/IL-17F and RORγt and in those without.

Overweight of Helios in the Regulatory T Cells within the CCR6$^+$CXCR3$^-$ CD4$^+$ Population

Human CCR6$^+$ T leukocytes of the peripheral blood are known to accommodate a subset of Tregs (31). Expression of signature genes characteristic for Tregs, *Foxp3*, Helios (coded by *IKZF2*), *IL2RA*, *CTLA4* and *CCR4* could be readily identified in the studied samples (Figures 3A, 4B). The key determinants of Tregs, *Foxp3* and *IL2RA*, were similarly expressed in RA patients and in healthy controls, and they had similar low expression and protein levels of the suppressive cytokine IL-10 (Figures 3A, 2B). Interestingly, the expression of *Foxp3* within individual patients was almost always discordant to the expression of the canonical Th17 regulator, *RORγt* (Figure 3B), and correlated negatively to *IL-23A* (r = –0.50, p = 0.02). Transcription pattern of signature cytokines *IL-17* and *IL-10* repeated the display of transcription factors (Figure 3B).

TGFβ and IL-1 signaling pathways are common for regulation of Th17 and Treg development (14). In RA patients, we found high expression of *IL1R1* and *TGFB2R* (Figure 3A). The transcriptional and protein levels of their ligands, *TGFB* (Figure 3A) and IL-1α and β (data not shown), were low and consequently led to low expression of the signal transducer and activator of transcription (STAT) family proteins downstream of TGFβ signaling (Figure 4A), despite repression of the TGFβ pathway inhibitor Smad7. Nuclear factors of activated T cells (NFAT1 and NFAT2), which induce expression of the *Foxp3* gene and function as transcriptional partners of Foxp3, were deregulated and predisposed to dysfunctional Tregs. The suppressors of cytokine signaling (SOCS) family maintaining stability of inducible Tregs (32,33) was also underexpressed in RA patients.

The expression of Helios (coded by *IKZF2*), a marker of thymus-derived Tregs, was higher in RA patients (Figures 3A, B). The overweight of Helios was accompanied by surface receptors *IL1R1* and T cell immunoreceptor with Ig and ITIM domains (*TIGIT*), important for suppressive function of Tregs (34–36). This created a remarkable disproportion in the transcriptional profile of Tregs within the CCR6$^+$CXCR3$^-$ CD4 cells of RA patients.

CCR6$^+$CXCR3$^-$ CD4$^+$ T Cells Have a Strong Follicular T-Helper (Tfh) Cell Profile

Circulating CXCR5$^+$CCR6$^+$ CD4$^+$ memory T cells have been identified as the population providing help to naive B cells and inducing polyclonal immunoglobulin (Ig) production (37). Flow cytometric analysis showed that CCR6$^+$CXCR3$^-$ CD4$^+$ cells had significantly larger subset of CXCR5$^+$ cells com-

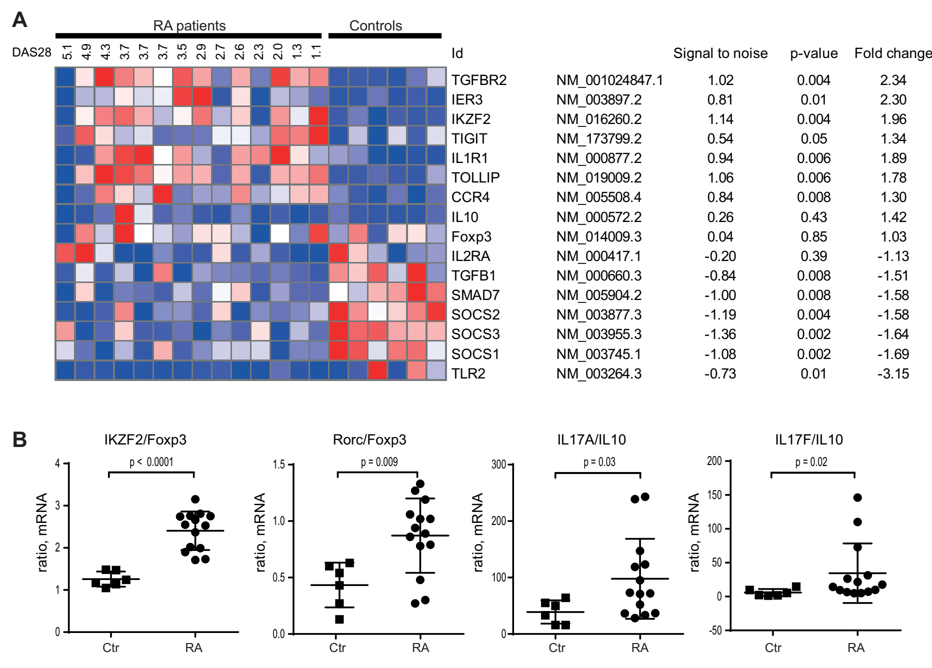

Figure 3. (A) Gene expression profiles of PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients and healthy controls. The differential expression levels for genes (rows) in patients or controls (columns) are shown. Normalized raw gene expression values from microarray experiments were used to identify differentially expressed genes involved in Th17 differentiation and function. DAS28, disease activity score based on 28 defined joints, is indicated for each RA patient. (B) Gene expression ratios for Th17- and Treg-associated genes.

pared with CCR6$^-$CXCR3$^-$ and CCR6$^-$CXCR3$^+$ CD4$^+$ cells (Figure 4A).

The transcriptional profile specific for Tfh cells was enriched in the isolated CCR6$^+$CXCR3$^-$ CD4$^+$ cells of RA patients compared with healthy controls. This result was recognized by high transcription of adaptor molecules *CXCR5*, *SLAMF*, inducible T-cell co-stimulator (*ICOS*) and *CTLA4*, facilitating their contact with germinal center B cells, and by transcription regulator *Bcl-6* (Figure 4B). The activated ICOS signaling was represented by higher expression of *ICOS* and changed expression of NFATc1 (gene *NFAT2*) and NFATc2 (gene *NFAT1*) mediating ICOS effects inside the cells. This result enhanced the transcription of *CXCR5* and *IL-21*, providing essential positive feedback for the differentiation of Tfh cells. Sufficient levels of IL-21 suppress transcription of *Blimp-1*, permitting upregulation of *Bcl-6* (Figures 4B, C). RA patients were also characterized by high production of CCL20, recruiting distant CCR6$^+$ T cells to maintain sufficient levels of IL-21, and of cytokines IL-31 and leukemia inhibitory factor (LIF) (Figure 4B), activating gp130 receptor of B cells essential for the germinal center reaction (38).

The phenotype of CD27$^+$CD45RA$^-$ memory CD4$^+$ T cells revealed the presence of CXCR5$^+$ and Bcl-6$^+$ subsets (Figures 4D, E). The volume of the Bcl-6$^+$ population within the memory T cells showed a tendency to correlate with the disease activity (Figure 4E), whereas no association with the serum levels of autoantibodies (RF and/or anti-CCP antibodies) was observed.

Clinical Remission and the Transcriptional Profile of CCR6$^+$CXCR3$^-$ CD4$^+$ Cells

To assess if the disease activity changes the transcriptional profile of CCR6$^+$CXCR3$^-$ CD4$^+$ cells, patients with active RA (DAS28 range 3.49–5.10, n = 7) and patients in clinical remission (DAS28 range 1.25–3.0, n = 7) were compared. The transcription of 109 genes showed a significant difference between RA patients with active disease compared with the healthy controls, and this number was reduced to 54 in RA patients in remission.

The transcriptional signature of Th17 cells of RA patients showed limited correlation to the clinical disease activity. The patients with active disease showed no significant difference from the patients in remission. The proinflammatory cytokine signature of Th17 cells with high levels of *IL-17F*, *IL-23A* and *TNF* expression was obvious both in active RA and in remission (Figure 5A). The expression of the transcriptional regulators *TBX21* and *JAK2*, characteristic for aggressive pathogenic Th17 phenotype, were maintained high during anti-TNF treatment in active RA and in remission (Figure 5A). The receptor pattern (*IL-1R1*, *TGFβR2* and *IL-23R*) essential for the differentiation of Th17 cells, and *IL-2* controlling the autocrine expansion, were similar in active disease and in clinical remission (Figure 5B).

The transcriptional profile of Tfh cells with characteristic transcription of *CXCR5*, *SLAMF-1* and *IL-21* was well preserved in RA patients in remission and was significantly higher compared with healthy controls (Figure 5C). These markers tended to be even higher in the patients with active RA. The difference in expression of the transcriptional regulator *Bcl-6* in RA patients in remission was no longer significant from the healthy controls. Consequently, CCR6$^+$CXCR3$^-$CD4$^+$ cells of RA patients in remission were recognized by somewhat lower expression of chemoattractant *CCL20* and lower expression of B-cell–activating cytokines *IL-31* and *LIF*.

DISCUSSION

The present study demonstrates that the peripheral blood CCR6$^+$CXCR3$^-$ CD4$^+$ cells of RA patients successfully treated with TNF inhibitors harbor pathogenic Th17 cells. The transcriptional and proteomic analysis revealed evident features of an aggressive proinflammatory phenotype with high expression of *IL-23R* and *GM-CSF*, and the combined upregulation of master transcription factors *RORγt* and

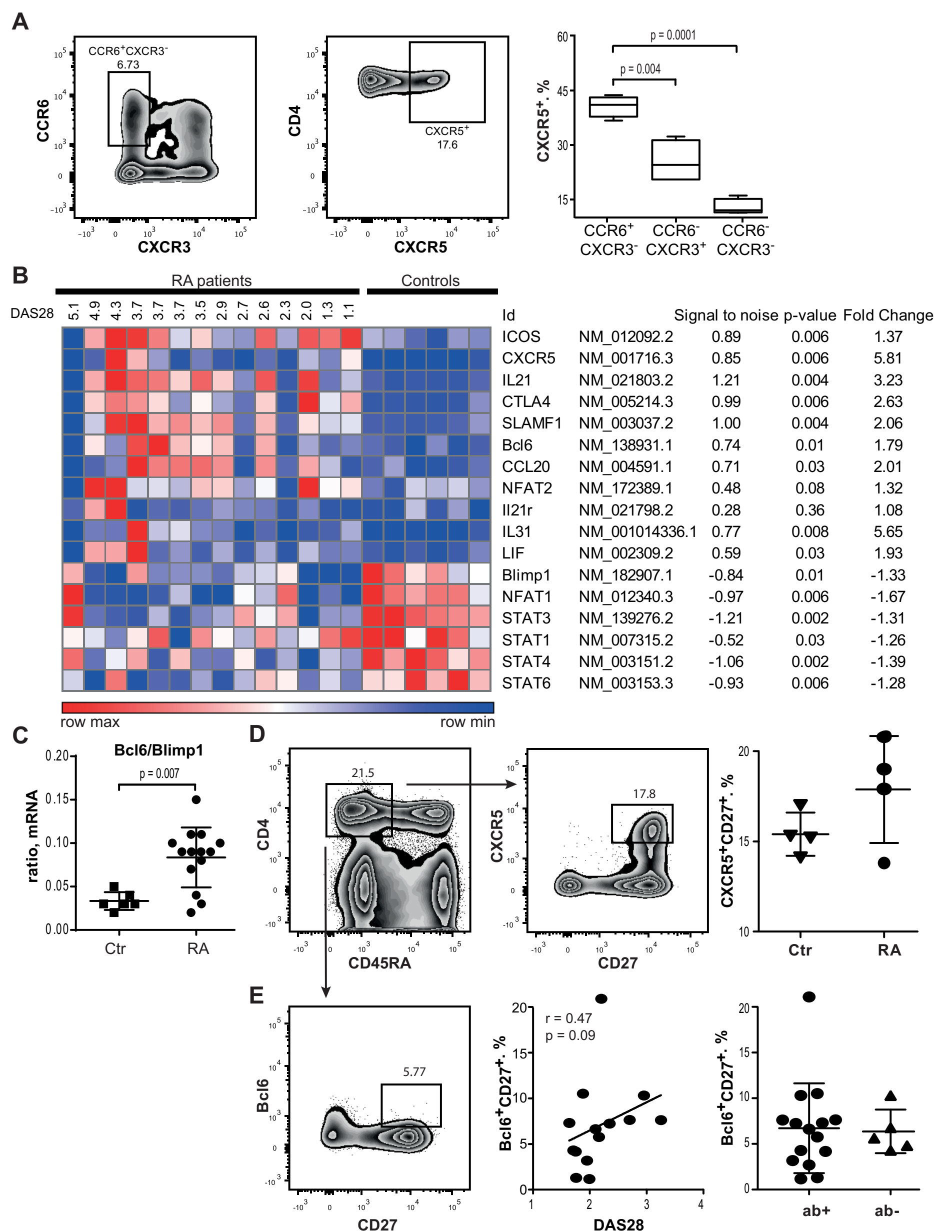

Figure 4. (A) Representative FACS plots illustrating CCR6/CXCR3-expressing CD4 cells and the CXCR5$^+$ population herein. CCR6$^+$CXCR3$^-$ CD4$^+$ cells had a significantly larger subset of CXCR5$^+$ cells compared with CCR6$^-$CXCR3$^-$ and CCR6$^-$CXCR3$^+$ CD4$^+$ cells (n = 4). (B) Gene expression profiles of PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients and healthy controls. The differential expression levels for genes (rows) in patients or controls (columns) are shown. Normalized raw gene expression values from microarray experiments were used to identify differentially expressed genes involved in Tfh differentiation and function. DAS28, disease activity score with 28 joint count, is indicated for RA patients. (C) The Bcl6/Blimp1 expression ratio in RA patients and healthy controls. (D) Flow cytometric analysis of CD27$^+$CD45RA$^-$ memory CD4$^+$ T cells' expression of CXCR5 in RA patients (n = 4) and healthy controls (n = 4). (E) Flow cytometric analysis of CD27$^+$CD45RA$^-$ memory CD4$^+$ T cells' expression of Bcl-6 in RA patients (n = 14). The size of the Bcl-6$^+$CD27$^+$ population (within CD45RA$^-$CD4$^+$) tended to correlate to DAS28 (r = 0.47, p = 0.09) in patients with autoantibodies (RF and/or anti-CCP), but showed no association with the presence of autoantibodies (ab$^+$ = patients with RF and/or anti-CCP antibodies).

TBX21 supported by high levels of signature cytokines IL-17A/IL-17F (39).

We could follow the complete transcriptional circuit of Th17 differentiation in RA patients enforced by active IL-1R1 and assured production of IL-21. The production of IL-21 is known to be significant, since it acts to induce amplification of differentiating Th17 cells and elicits the expression of *IL-23R* (17,40). This exposure to IL-23 stabilized the Th17 phenotype in RA patients and, together with IL-21, induced production of another proinflammatory cytokine, IL-22 (41). The late phase of Th17 development is associated with the expression of the regulatory cytokines IL-10 and IL-24 (16,21). IL-24 was overexpressed in Th17 cells of RA patients and, together with the increased IL-2 and CD62L, supported formation of memory Th17 cells in the studied RA patients with active disease or in remission. It is important to acknowledge that expression of the dominating effector cytokine had significant intraindividual variations and could be represented by IL-22, IL-23, IL-2, TNF or GM-CSF. This result might reflect heterogeneity of molecular mechanisms engaged in the formation and maintenance of the Th17 profile in RA. Additionally, the pathogenic profile of Th17 cells showed only a limited correlation with the disease activity measured clinically. It was statistically indistinguishable between the patients in remission and those who had clinical signs of active RA. Th17 cells of patients in remission maintained high *IL-23R* and *IL-1R1* expression, the receptors essential for their differentiation (42), and for the production of proinflammatory IL-17F, IL-23, IL-21 and TNF.

Th17 and Tregs use common intracellular mechanisms for their differentiation and present a complicated functional interplay. The persistent enrichment of CCR6$^+$CXCR3$^-$ CD4$^+$ cells with Th17 transcriptional profile has been recently described as a result of transdifferentiation of Tregs in autoimmune arthritis (23). This process of conversion of Tregs into Th17 cells is proposed to be mediated by synovial fibroblasts and is characterized by high expression of CCR6, IL-23R and CCL20. Experimentally, conversion of

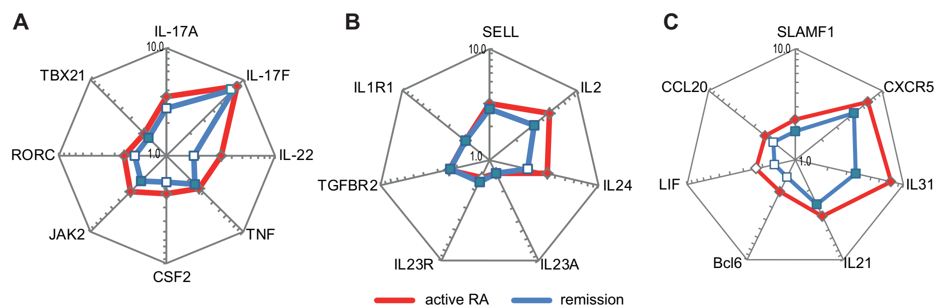

Figure 5. Fold-change values of gene expression of PMA-ionomycin–stimulated CCR6$^+$CXCR3$^-$ CD4$^+$ cells from RA patients in remission (blue, n = 7) and with active disease (red, n = 7), respectively, versus healthy controls (n = 6). (A) Cytokines and transcription factors characteristic for Th17; (B) receptors essential for Th17 differentiation, and IL-2 controlling proliferation; (C) cytokines, transcription factors, and receptors characteristic for Tfh cells. Filled symbols indicate significant difference to controls.

Tregs to Th17 cells was accomplished upon IL1b, IL-6 and IL-23 exposure and was recognized by loss of Foxp3 (43–45). The studied CCR6$^+$CXCR3$^-$ T cells of RA patients had a relative deficiency in transcription of the *Foxp3*, *CTLA4* and *IL2RA* genes and a low production of suppressor cytokine IL-10, strongly suggesting altered Treg functions. Simultaneously, a remarkable overexpression of transcription factor Helios was found, which implied a recent thymic origin of the cells. Helios, in combination with surface receptors IL1R1 and TIGIT, has been shown to be important for suppressive functions of Tregs (35,36). The loss of Foxp3 and enrichment of the Helios$^+$Foxp3$^-$ T-cell subset may represent an intermediate phenotype deprived of suppressive function or it may be an aberrant cell subset with stimulatory and autoreactive properties, as it has been described for early Heios$^+$Foxp3$^-$ thymocytes in mice (46). Production of IL-2 and TNF cytokines is recognized as an important stimulatory effect of Tregs applied on all types of Th cells, including Th17. These TNF- and IL-2–dependent pathways were obviously functional in the studied RA patients despite the long-term neutralization of TNF and could have interfered with Treg activity (47) and supporting further a continuous accumulation of Th17 cells in RA patients during anti-TNF treatment.

IL-21 is considered essential for the formation of Tfh and as one of the major means of communication between Tfh and germinal center B-cells (48,49). In patients with early RA, serum levels of IL-6 and IL-21 help distinguishing RA patients with established disease from those with undifferentiated arthritis (50). CCR6$^+$CXCR3$^-$ CD4$^+$ cells of RA patients had the phenotype of functional Tfh with high transcription of *CXCR5* accompanied by *ICOS* and *IL-21* leading to upregulation of transcriptional repressor *Bcl-6*. Bcl-6 is the major transcription factor for Tfh differentiation, which is shown to be induced by a number of complementary ways, including signaling through ICOS, and IL-6, IFN and IL-21 receptors activating JAK/STAT pathways (51). During anti-TNF treatment, the RA patients maintained high JAK2, presumably supporting transcription of *Bcl-6*. Additionally, high IL-2 may facilitate *Bcl-6* transcription in CCR6$^+$CCR3$^-$ CD4 T cells by inhibiting its fundamental repressor Blimp-1 (52).

CXCR5$^+$CCR6$^+$CCR3$^-$ CD4 cells of RA patients produced LIF and IL-31, the cytokines activating the IL-6 signaling transducer gp130 of B-cells, aiding differentiation of naive B cells into immunoglobulin-producing plasmablasts (37). Constant activation of gp130 has been experimentally connected to spontaneous development of erosive arthritis in mice (53,54). In our limited patient material, we could not see an association between the transcriptional profile of Tfh and serum autoantibody levels, which may be partly explained by the dominance of seropositive patients with long-term and destructive disease.

CONCLUSION

Taken together, this study presents evidence that CCR6$^+$CXCR3$^-$ CD4$^+$ cells of RA patients harbor pathogenic Th17 cells potentially transdifferentiated from dysfunctional Tregs and maintain a source of autoimmunity in RA patients during anti-TNF treatment.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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