Aberrant expression of USF2 in refractory rheumatoid arthritis and its regulation of proinflammatory cytokines in Th17 cells

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IL-17-producing Th17 cells are implicated in the pathogenesis of rheumatoid arthritis (RA) and TNF-α, a proinflammatory cytokine in the rheumatoid joint, facilitates Th17 differentiation. Anti-TNF therapy ameliorates disease in many patients with rheumatoid arthritis (RA). However, a significant proportion of patients do not respond to this therapy. The impact of anti-TNF therapy on Th17 responses in RA is not well understood. We conducted high-throughput gene expression analysis of Th17-enriched CRC6^+CXCR3^+CD45RA^−CD4^+ T (CRC6^+ T) cells isolated from anti-TNF–treated RA patients classified as responders or nonresponders to therapy. CRC6^+ T cells from responders and nonresponders had distinct gene expression profiles. Proinflammatory signaling was elevated in the CRC6^+ T cells of nonresponders, and pathogenic Th17 signature genes were upregulated in these cells. Gene set enrichment analysis on these signature genes identified transcription factor USF2 as their upstream regulator, which was also increased in nonresponders. Importantly, short hairpin RNA targeting USF2 in pathogenic Th17 cells led to reduced expression of proinflammatory cytokines IL-17A, IFN-γ, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as transcription factor T-bet. Together, our results revealed inadequate suppression of Th17 responses by anti-TNF in nonresponders, and direct targeting of the USF2-signaling pathway may be a potential therapeutic approach in the anti-TNF refractory RA.

Significance
Identifying signaling pathways contributing to resistance to anti-TNF therapy in rheumatoid arthritis is crucial for the development of new therapeutic strategies for refractory rheumatoid arthritis. Th17 cells, a subset of proinflammatory CD4^+ T cells, are implicated in the pathogenesis of the disease. We analyzed the gene expression profiles of Th17-enriched CD4^+ T cells in anti-TNF–treated patients with rheumatoid arthritis and found that the elevated expression levels of transcription factor USF2 in anti-TNF refractory patients were associated with increased proinflammatory signaling of Th17 cells. USF2–knockdown experiments in Th17 cells revealed that USF2 promotes the pathogenicity of Th17 cells. These findings have implications for the development of new therapeutic strategies for refractory rheumatoid arthritis.


The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2007935117/-/DCSupplemental.


Rheumatoid arthritis (RA) is a chronic autoimmune disorder affecting joints (1). Cytokines are involved in the pathogenesis of RA, including tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL-17, IL-23, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (2–4). TNF-α is primarily produced by activated macrophages and monocytes, although it may also be produced by lymphocytes and other cell types including T helper 17 cells (Th17) (3, 5–7), an IL-17–producing CD4^+ T cell subset which was first reported in murine autoimmune models in 2005 (9, 10). As one of the major proinflammatory cytokines present in the rheumatoid joint, TNF-α has proven to be a good therapeutic target for RA therapy, and TNF-α inhibitors effectively block disease progression and improve physical function (8). Th17 cells have been linked to autoimmune diseases including RA, multiple sclerosis, systemic lupus erythematosus, psoriasis, inflammatory bowel disease, and Crohn’s disease (11, 12). Increased frequency of Th17 cells and elevated IL-17 levels have been found in the peripheral blood of RA patients (13, 14). The increased frequency of Th17 cells correlates with the number of swollen joints and serum levels of C-reactive protein (15) and IL-17. Th17 cells in inflamed joints in RA orchestrate the chronic inflammation by stimulating fibroblast-like synovocytes to produce GM-CSF and expand proinflammatory-secreting synovial-resident innate lymphoid cells (16). When first discovered, Th17 cells were considered a homogenous proinflammatory population (9, 10). Shortly thereafter, an antiinflammatory subset of Th17 cells that coproduced IL-10 was identified (17), while proinflammatory/pathogenic Th17 cells are shown to express higher levels of IFN-γ (17–19). Therapeutic studies also reveal the complexity of Th17 function. Anti–IL-17 therapy ameliorates psoriasis, but blocking the IL-17–signaling pathway in Crohn’s disease is either ineffective or exacerbates diseases (20–22). Thus, it is not only important to determine Th17 cell frequency and IL-17 levels in patients with RA, but also to evaluate the proinflammatory capacity of Th17 cells (18, 19). In juvenile idiopathic arthritis, IFN-γ–secreting Th17 cells are highly enriched in the synovial fluid (23), and IFN-γ–negative Th17 cells can be converted to IFN-γ–secreting Th17 cells under conditions of the disease flare (24). In adult RA patients, studies indicate the migration of IFN-γ–secreting Th17 cells to synovial tissue of inflammation (25). These findings suggest the association of proinflammatory Th17 cells in RA pathogenesis and inhibition of their function is a therapeutic approach worthy of exploration.

Upon T cell receptor activation, IL-6 and TGF-β1 induce nonpathogenic Th17 differentiation, while the cytokine combination of IL-1β, IL-6, and IL-23 leads to pathogenic Th17
TNF-α promotes Th17 differentiation in RA via inducing stromal cells and monocytes to secrete proinflammatory cytokines IL-1β, IL-6, etc. (27, 28). A recent clinical study has reported that TNF-α inhibitor infliximab reduces the frequency of peripheral Th17 cells and IL-17 level in patients with RA (15), which implies a direct or indirect upstream therapeutic effect of anti-TNF-α on Th17 differentiation. In other diseases, TNF-α and IL-17 have been shown to have synergistic effects to amplify proinflammatory signals. In psoriasis both TNF-α and IL-17 are overexpressed in the skin lesions, and they act synergistically to affect cytokine production (29). An in vitro study of intervertebral disk cells also demonstrated that TNF-α and IL-17 synergistically facilitate inflammatory mediator release (30). Gene expression profiling of CCR6+CXCR3+CD4+ T cells from anti–TNF-α–treated RA patients, which are enriched for Th17 cells, found that these treated patients still display enhanced gene expression of IL17, RORC, IL22, and IL23R compared to healthy controls (31). A proof-of-concept clinical study has shown that the combination of anti–IL-17 and anti-TNF treatment effectively reduces disease activity in RA patients with inadequate response to anti-TNF treatment alone (32). Taken together, these studies indicate that the TNF-α and IL-17–Th17 pathways do not completely converge in RA pathogenesis and that the IL-17/Th17 pathway is a nonredundant therapeutic target for these diseases.

A lack of response to TNF-α inhibitors in a significant portion of patients is a serious problem in clinical rheumatology (33, 34). Investigation of responders and nonresponders to anti-TNF therapy can help to identify the pathways and key regulatory genes involved in refractoriness to the treatment. Here, we study the gene expression of Th17–enriched CCR6+CXCR3+ memory CD4+ T cells from responders and nonresponders to anti-TNF therapy in RA patients as well as Th17 cells induced in vitro. We identified the transcription factor USF2 to fuel-activated proinflammatory signaling pathways in anti-TNF refractory patients and experimentally confirmed the role of USF2 to control the expression of proinflammatory cytokines in pathogenic Th17 cells.

Results
Enrichment of Th17 Subsets in CD4+CCR6+CXCR3+ Cells. The expression of the CC chemokine receptor CCR6 is strongly correlated with both mouse and human Th17 cells (35, 36) and thus has been used as a cell surface marker to enrich Th17 cells given that there are no unique cell surface markers available for the Th17 subset. Based on the differential expression of chemokine receptors and other cell surface antigens, we utilized antibody-conjugated magnetic beads by negative selection to deplete non–CD4+ T cells by negative selection and to isolate Th17–enriched CCR6+CXCR3+ memory CD4+ T cells by positive selection (see Materials and Methods) (SI Appendix, Fig. S1 A and B). Cells isolated in this manner are only bound by anti-CCR6 antibody to minimize unwanted manipulation and are hereafter referred to as CCR6+ T cells. To assess the efficiency of this approach for Th17 enrichment, we isolated total memory CD4+ T (mCD4) cells and CCR6+ T cells from the blood of four healthy donors and stained cells for the intracellular production of IL-17, IFN-γ, IL-10, and GM-CSF (Fig. 1). Compared to mCD4 cells, the Th17 frequency in CCR6+ T cells was increased by 7- to 12-fold (Fig. 1 A and B), and Th1 (IL-17+IFN-γ−) frequency was reduced by ~70% (Fig. 1 A and C). The Th17/Th1 ratio was increased from 1.8/7 in mCD4 cells to 3.1/1 in CCR6+ cells with a fold change of 26.7. It has been reported that CXCR3 is rapidly induced in naïve T cells upon activation, and its expression remains high on IFN-γ–secreting Th1 and IFN-γ− Th17 cells (26, 37). However, IFN-γ+ Th17 cells were still enriched in CXCR3− CCR6+ T cells (Fig. 1 A and C and SI Appendix, Fig. S1B), demonstrating that, like Th1 cells, not all IFN-γ+ Th17 cells express cell surface CXCR3. Analysis of IL-10 and GM-CSF secretion in Th17 cells showed that the IL-10−, IL-10+, GM-CSF−, and GM-CSF+ Th17 subsets were also enriched in CCR6+ T cells. Thus, the CCR6 selection and CXCR3 depletion effectively enriched various Th17 subsets from peripheral blood.

Distinct Gene Expression in Responding and Nonresponding RA Patients to Anti-TNF Therapy. To assess the difference in gene expression of CCR6+ T cells between RA patients who responded or did not respond to anti-TNF treatment, we isolated CCR6+ T cells from six responding patients, seven nonresponding patients, and six healthy controls (Table 1). CCR6+ T cells were either activated with phorbol 12-myristate 13-acetate (PMA) / ionomycin for 4 h or left untreated and used as unstimulated controls. qPCR analysis of the activated cells showed that the expression levels of IL17A, IL17F, and IFNG were not different among these three groups. The CSF2 levels in both responding and nonresponding patients were elevated with nonresponders showing the trend of highest expression. The levels of TNF messenger RNA (mRNA) in the nonresponders but not in the responders were significantly higher compared to healthy controls (Fig. 24). We also subjected the cells to the nCounter Gene Expression Analysis using the custom CodeSet HuTH17 that detects 418 genes associated with human T-helper cell differentiation and activation (19). For the 19 PMA/ionomycin stimulated and 19 unstimulated CCR6+ T cell samples we analyzed, 397 of 418 genes showed maximum expression levels across all samples above background and were selected for further analysis. The expression levels of IL17A, IL17F, IFNG, CSF2, and TNF in stimulated CCR6+ T cells were consistent with the results obtained with qPCR analysis (SI Appendix, Fig. S2). Supervised clustering (Fig. 2B) and principal component analysis (PCA) (Fig. 2C) of the 397-gene expression profiles of the 38 individual samples demonstrated distinct transcriptional characteristics between stimulated and unstimulated cells; in contrast, the difference among responding, nonresponding patients, and healthy controls was relatively minor. Nevertheless, the PCA plot clearly segregated the nonresponders from healthy controls in the stimulated samples. To better reveal the difference among sample groups, we analyzed stimulated and unstimulated samples independently. Here we focused on the genes with one-way ANOVA P value < 0.05 among the three sample groups of either stimulated or unstimulated cells. Based on this restriction, 172 and 74 genes were selected for the stimulated (SI Appendix, Table S1) and unstimulated samples (SI Appendix, Table S2), respectively. PCA showed that healthy controls were well separated from RA patients for both stimulated and unstimulated CCR6+ T cells, while responders were well separated from nonresponders for unstimulated cells and, to a lesser extent, for stimulated cells (Fig. 2D). These results demonstrated that differential gene expression in CCR6+ T cells not only distinguished patients from healthy donors but also responders from nonresponders to anti-TNF treatment.

Enhanced Proinflammatory Signaling in Nonresponding Patients. In unstimulated CCR6+ T cells, 16 of the 397 genes were differentially expressed between nonresponders vs. responders with 2 up-regulated and 14 down-regulated in the nonresponding patients. The PDIL, LAG3, and CTLA4 genes, which encode checkpoint inhibitors and are associated with T cell exhaustion (38, 39) and FoxP3+ CD4+ Treg-mediated suppression (40), were among the 14 down-regulated genes in nonresponding patients (Fig. 3A). Yet, PDIL, LAG3, and CTLA4 displayed different patterns of relative expression among healthy controls and RA patients (Fig. 3B). No significant difference of PDIL expression was observed between healthy controls and nonresponding patients, but its expression was up-regulated in responding patients. For LAG3, nonresponding patients displayed repressed expression
compared to healthy controls and responding patients, while its expression was comparable between the two latter groups. The expression pattern of \textit{CTLA4} was more similar to that of \textit{PD1L} than that of \textit{LAG3}, with it not showing a reduced expression in nonresponding patients compared to healthy controls. The Ingenuity Pathway Analysis (IPA) upstream regulator analysis on the 16 DE genes identified the upstream regulator NFATC2 inhibited in nonresponders (Fig. 3C). NFATC2 is an intrinsic negative regulator of T cell activation (41) and facilitates Treg induction and function (42). The reduced expression of \textit{LAG3} and \textit{CTLA4} and inactivated NFATC2-signaling pathway in the CCR6$^+$ T of nonresponders suggested a lower Treg activity compared to responders. In stimulated CCR6$^+$ T cells, 35 genes were differentially expressed between nonresponders and responders (Fig. 3D). Among the top six genes up-regulated in nonresponders with a fold change above 2, four genes encode proinflammatory cytokines IL-31 (43), IL-22 (44), IL-24 (45), and GM-CSF; one encodes T cell growth factor IL-2. While the expression of \textit{IL31}, \textit{IL22}, \textit{IL24}, and \textit{CSF2} was increased in nonresponding patients, their expression levels between responding patients and healthy controls were comparable (Figs. 2A and 3E). Among the DE genes, \textit{IL22}, \textit{CSF2}, \textit{IL2}, and \textit{SLAMF1} are known pathogenic Th17 signature genes (19). The IPA upstream regulator analysis of the 35 DE genes showed that IL-1$\beta$ and NF-$\kappa$B were the top two activated upstream regulators; GM-CSF was also among the activated upstream regulators. In contrast, butyric acid (46, 47) and curcumin (48–50), which activate anti-inflammatory pathways, were among the inhibited upstream regulators (Fig. 3F). These results showed that the anti-TNF treatment was inadequate to down-regulate the abnormally elevated immune responses in the CCR6$^+$ T cells of nonresponding patients compared to that of responders.

**Enriched Pathogenic Th17 Gene Signature in Nonresponders.** To interrogate whether the enhanced proinflammatory activity in the CCR6$^+$ T cells of nonresponders was related to the pathogenicity of Th17 cells, we performed gene set enrichment analysis (GSEA) to cross-examine the similarities in signature genes between mouse pathogenic Th17 cells and the CCR6$^+$ T cells of responders and nonresponders. Since many of the Th17-associated genes were primarily expressed in stimulated cells (SI Appendix, Fig. S3), we performed the GSEA with the gene expression profiles
while the overlap in the down-regulated gene signatures was less
nonresponders and responders, respectively. The up-regulated
genes were up-regulated and 41 down-regulated in nonresponding
sponders vs. healthy controls. Compared to healthy controls, 73
of stimulated CCR6+ T cells. First, we identified the DE genes
between nonresponders vs. healthy controls and between re-
responders vs. healthy controls. Compared to healthy controls, 73
genes were up-regulated and 41 down-regulated in nonresponding
patients, whereas only 17 genes were up-regulated and 35 down-
regulated in responding patients (Fig. 4A). These four gene sets
formed the up-regulated and down-regulated gene signatures of
nonresponders and responders, respectively. The up-regulated
gene signature of responders was smaller than and largely in-
cluded in that of nonresponders, with 16 out of the 17 signature
genes present in the up-regulated gene signature of nonresponders,
while the overlap in the down-regulated gene signatures was less
(Fig. 4B and SI Appendix, Tables S3 and S4). Treating murine
naive CD4+ T cells with TGF-β3/IL-6 or IL-1/IL-6/IL-23 cytokine
mixtures induces pathogenic Th17 differentiation, whereas TGF-
β1/IL-6 induces nonpathogenic Th17 cells, and the whole-genome
microarray data on these murine Th17 populations are publicly
available (18). Thus, the four gene signatures of nonresponders
and responders were analyzed for gene set enrichment by being
compared to murine TGF-β3/IL-6–induced Th17 cells (patho-
genic) vs. TGF-β1/IL-6–induced Th17 cells (nonpathogenic) (scen-
ario 1); and IL-1/IL-6/IL-23–induced Th17 cells (pathogenic) vs.
TGF-β1/IL-6–induced Th17 cells (nonpathogenic) (scenario 2). GSEA
results demonstrated that genes up-regulated in nonresponding
patients were enriched in the mouse pathogenic vs. nonpathogenic
Th17 comparison in both scenarios, but genes up-regulated in
responding patients were not enriched (Fig. 4C). The enriched
leading-edge genes of nonresponding patients identified by the two
comparative scenarios were largely overlapping (Fig. 4D). Genes
down-regulated in nonresponding patients or in responding pa-
tients were both enriched in the mouse pathogenic vs. nonpatho-
genic Th17 cell comparison (SI Appendix, Fig. S4A), and the
enriched genes were largely overlapping between the nonre-
sponder vs. mouse Th17 and responder vs. mouse Th17 compar-
sions for both scenarios (SI Appendix, Fig. S4B). We conducted
these analyses to probe the key genes and pathways that were
potentially involved in resistance to anti-TNF treatment in RA.
These results demonstrated that the gene expression disparity in
CCR6+ T cells between anti-TNF responding and nonresponding
patients relative to pathogenic Th17 cells was found in up-regulated
but not down-regulated signature genes, and the up-regulated
leading-edge genes of CCR6+ T cells in nonresponders could be
utilized to identify the plausible pathways that modulated the
pathogenicity of Th17 cells to help establish the resistance to anti-
TNF therapy in RA.

Identification of USF2 as the Upstream Regulator of the Refractory Gene Signature. Merging the leading-edge genes listed in Fig. 4D, we obtained a molecular signature of CCR6+ T cells, which comprised 23-enriched up-regulated genes of nonresponding patients related to Th17 pathogenicity in contrast to responding patients. The expression of these up-regulated signature genes was progressively increased from healthy controls to responding and nonresponding patients (Fig. 5A). Comparing this gene signature to the DE genes between responders vs. healthy controls (SI Appendix, Table S4), we found five overlapping genes (CRL4A, IKZF2, IL1R1, IL2, and SLAMF1) (Fig. 5A, Lower). To ensure that the signature genes we identified were unique for nonresponders, we removed the five overlapping genes and formed an anti-TNF refractory gene signature with the remaining 18 signature genes (Fig. 5 A, Upper). In the next step, we interrogated the obtained refractory gene signature for upstream transcription factors involved in nonresponsiveness to anti-TNF treatment with the Enrichr ENCODE TF CHIP-SEQ 2015 analysis (51, 52). The analysis indicated USF2 (upstream stimulatory factor 2), a basic helix–loop–helix–leucine–zip transcription factor, as the top predicted upstream transcription factor with 10 of the 18 refractory signature genes enriched in this pathway. USF2 was also the only predicted upstream transcription factor with an adjusted P value < 0.05 (Fig. 5B and SI Appendix, Table S5). We conducted qPCR analysis on USF2 in these RNA samples because it was not included in the CodeSet HuT117. Short-term (4 h) PMA/ionomycin stimulation dramatcally reduced the mRNA levels of USF2 (Fig. 5C). Ionomycin is a calcium ionophore and induces calcium influx in treated cells (53). PMA activates Ca2+-dependent protein kinase C (54). Thus, PMA and ionomycin synergize to activate protein kinase C. Repressed expression of USF2 in PMA/ionomycin-treated T cells indicates that USF2 may be negatively regulated by protein kinase C. We then compared the expression levels of USF2 between responding and nonresponding patients. qPCR analysis showed that the average mRNA level of USF2 was higher in nonresponding patients but not in responding patients compared to healthy controls in the unstimulated cells, while no significant difference was observed in stimulated cells (Fig. 5D). These results suggest an association between enhanced expression of USF2 and increased pathogenic Th17 signaling in nonresponders to anti-TNF therapy in RA, as well as that a strong cell stimulation signal such as PMA/ionomycin stimulation could rapidly shut off or override the function of USF2.

USF2-Dependent Proinflammatory Cytokine Expression in Pathogenic Th17 Cells. We then tested how USF2 regulates the differentiation of pathogenic Th17 cells induced from naive CD4+ T cells with IL-1β, IL-6, IL-23, and TGF-β cytokine mixture treatment in the presence of anti-CD3/28 stimulation. The levels of USF2 transcripts were comparable among ex vivo isolated peripheral blood mononuclear cells (PBMC), total CD4+ T cells, and naive CD4+ T cells (SI Appendix, Fig. S5A). We observed increased expression of USF2 while subjecting naive and memory CD4+ T cells to the pathogenic Th17 differentiation condition (SI Appendix, Fig. S5B). To investigate whether USF2 regulates pathogenic Th17 development, we used a lentiviral vector to introduce a short hairpin RNA (shRNA) targeting USF2 or a scramble control shRNA into CD4+ T cells during differentiation of pathogenic Th17 cells. In this way, we generated the USF2-knockdown pathogenic Th17 cell lines and their corresponding mock controls. In the control cell lines, we observed an elevated expression of USF2 on days 5 and 7 after subjecting naive CD4 T cells to pathogenic Th17 polarization condition (Fig. 6A). BCL6, NOLC1, NOP16, and PTRH2 are downstream targets of USF2 in
human liver cancer cell line HepG2 cells (Fig. 5B and SI Appendix, Table S5), and the expression levels of these genes in nonresponders were elevated compared to healthy controls (Fig. 5A) as well as compared to responders (Fig. 5D). We found, in USF2-knockdown Th17 cells, the expression of NOLC1, NOP16, and PTHR2 but not BCL6 was repressed on day 7 but not on day 5 after subjecting CD4+ T cells to pathogenic Th17 polarization conditions (Fig. 6B), which suggests that 1) although the expression of USF2 was induced at an early stage during pathogenic Th17 differentiation, its regulatory function manifested at a later stage; and 2) the respective USF2-signaling pathways in HepG2 and Th17 cells were likely largely but not completely overlapping. We then looked into Th17-associated cytokines, transcription factors, etc., in USF2-knockdown cells. On day 5, we observed a minor but significant reduction of TBX21 expression (Fig. 6, Left). However, after an additional 2 d, not only the reduced expression of TBX21 (P = 0.007) was sustained, the expression of IL17A (P = 0.0004), IFNG (P = 0.029), IL22 (P = 0.024), TNF (P = 0.011), and CSF2 (P = 0.056) was also effectively inhibited without reducing the expression of IL23R, RORC, and STAT3 (Fig. 6 C, Right). These results suggest that USF2 affects T-bet-mediated proinflammatory program and works downstream or independently of ROR-γt, Stat3, and IL-23 receptor during Th17 development. USF2 is critical to sustaining proinflammatory cytokine expression at late stage of Th17 development.

**Discussion**

Through studying the gene expression profiles of CCR6+ T cells from patients responding and not responding to anti-TNF therapy, we found that these cells in nonresponders displayed the gene expression feature of pathogenic Th17 cells, and computational
analysis identified transcript factor USF2 as being responsible for this feature. The gene expression of USF2 in CCR6+ T cells from nonresponders was elevated. A previous study has reported USF2-dependent RORC (RORγt in the publication) expression in RORC promoter reporter constructs transfected into HepG2, HeLa, and Jurkat cells and showed that even though small interfering RNA inhibition of USF2 in induced Th17 cells led to reduced expression of RORC, IL17A expression was not reduced (55). However, we found USF2 silencing in induced Th17 cells did not affect RORC expression as well as STAT3 expression, which encodes the key transcription factors RORγt and Stat3 for Th17 differentiation. Instead, USF2 silencing led to reduced gene expression of transcription factor T-bet and proinflammatory cytokines IL-17A, IFN-γ, IL-22, TNF-α, and GM-CSF. Interestingly, the inhibition of TRX21 in USF2-knockdown cultures occurred already on day 5 of the pathogenic Th17 differentiation and preceded the suppression of these effector proinflammatory cytokine genes that only happened at late stage (day 7), which suggests a T-bet–dependent mechanism of prolonged sustainment of proinflammatory cytokines in pathogenic Th17 cells. Taken together, our results suggest that USF2 is dispensable for the initiation of pathogenic Th17 differentiation, but it plays an important role in sustaining inflammatory cytokine expression in pathogenic Th17 cells.

Among the CCR6+ T cells in this study, only about 20% of them secreted IL-17, and high levels of expression in this study were easily reached upon short-term activation by PMA/ionomycin. CCR6, a CC chemokine receptor protein that belongs to family A of the G protein-coupled receptor superfamily, is not restricted to Th17 cells (26, 56, 57). It is also expressed by a subset of Tregs (58). Thus, these CCR6+ T cells were not only enriched for Th17 cells but likely for CCR6+ Tregs as well. Expression of CCR6 on Tregs facilitates the recruitment of Tregs to inflamed tissue without affecting their suppressive functions (58). Comparing the expression profile of unstimulated CCR6+ T cells of anti-TNF nonresponders to that of responsive patients, we found the elevated expression of LAG3 and CTLA4 (Fig. 3 A and B). LAG-3 and CTLA-4 are expressed by FoxP3+ CD4+ Tregs and mediate Treg suppression. High expression of CTLA-4 positively correlates with suppressive activity of Tregs (40). Hence, the increased expression of LAG3 and CTLA4 in responders suggests higher Treg activity in patients responsive to anti-TNF therapy compared to nonresponders.

Consistent with this observation, the IPA upstream regulator analysis using the differentially expressed genes in CCR6+ T cells between responding vs. nonresponding patients showed that the butyric acid and curcumin pathways were down-regulated in nonresponding patients. Butyric acid is a microbial metabolite found in many food products, especially milk products. Butyrate, the salt format of butyric acid, promotes Treg generation and inhibits Th17 differentiation and is found to ameliorate autoimmune diseases. This suggests that the butyric acid and curcumin pathways may play a role in regulating Treg and Th17 differentiation.
uveitis and colitis in animal models (46, 47, 59). Curcumin is a chemical extracted from *Curcuma longa* plants used in cooking and cosmetics. It has antioxidant and antiinflammatory properties (49). Curcumin has been shown to inhibit Th17 differentiation both in animal models (60) and in vitro human studies (61). On the other hand, multiple proinflammatory pathways including the IL-1β, NF-κB, and GM-CSF – signaling pathways were activated in nonresponding patients. Thus, our results showed inadequate suppression of proinflammatory immune responses by anti-TNF in refractory patients, and the USF2-signaling pathway may be responsible for the resistance to treatment. USF2 is ubiquitously expressed and participates in embryonic development, brain function, metabolism (62), and possibly in hematopoietic stem cell development (63). Several studies suggest that USF2 also contributes to cancer development (64, 65). Yet little is known about the function of USF2 in regulating the immune system, not to mention in the context of pathogenic Th17 regulation. Further study of the role of USF2 in pathogenic Th17 function may shed a light on disease pathogenesis and lead to development of novel therapeutic interventions for refractory RA.

**Materials and Methods**

**Human Subjects.** Blood samples were obtained from 6 healthy donors and 14 patients with established RA. The study was approved by the Regional Ethics Board in Gothenburg, Sweden (Dnr 633-07), and informed written consent was obtained from all participants. The patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (66). One of the 14 patients was excluded from the study due to inadequate in vitro stimulation for gene expression analysis. All of the remaining 13 RA patients were female and obtained infliximab injection intravenously (i.v.) at a dose of 200 mg every 8 wk. Twelve patients were also treated with methotrexate (7.5–25 mg/wk), and the remaining patient was treated with mycophenolate mofetil (2 g/d). Response to treatment was determined according to DAS28 criteria, with DAS28 < 2.8 as responding and ≥ 2.8 as nonresponding at the time of blood sampling. All of the patients were refractory to treatment with conventional disease-modifying drugs, such as methotrexate and combination of methotrexate with hydroxychloroquine. Two of the patients were previously treated with a different anti-TNF drug (etanercept). Six patients responded to treatment including the mycophenolate mofetil-treated one, and the other seven were nonresponders. Four patients of each group were ACPA-positive. Mean total dose of infliximab for the responders and nonresponders were 8,367 and 8,257 mg, respectively. The control group comprised six healthy subjects with no report of any autoimmune disease or the use of any pharmacological drug. Blood samples from healthy donors were also obtained from the Partners Multiple Sclerosis Center at Brigham and Women’s Hospital under Institutional Review Board (IRB) Protocol 1999P010435 for cell surface antigen and intracellular cytokine-staining assays and in vitro Th17 cell differentiation analysis.

**Cell Isolation and Stimulation.** PBMC were isolated with Ficoll-Paque PLUS (GE Healthcare) separation. Total CD4+ T cells were isolated from PBMC with the EasySep Human CD4+ T Cell Enrichment Kit (StemCell Technologies, catalog number 19052) following manufacturer’s instruction. Naive CD4+ T cells was obtained from all participants. The patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (66). One of the 14 patients was excluded from the study due to inadequate in vitro stimulation for gene expression analysis. All of the remaining 13 RA patients were female and obtained infliximab injection intravenously (i.v.) at a dose of 200 mg every 8 wk. Twelve patients were also treated with methotrexate (7.5–25 mg/wk), and the remaining patient was treated with mycophenolate mofetil (2 g/d). Response to treatment was determined according to DAS28 criteria, with DAS28 < 2.8 as responding and ≥ 2.8 as nonresponding at the time of blood sampling. All of the patients were refractory to treatment with conventional disease-modifying drugs, such as methotrexate and combination of methotrexate with hydroxychloroquine. Two of the patients were previously treated with a different anti-TNF drug (etanercept). Six patients responded to treatment including the mycophenolate mofetil-treated one, and the other seven were nonresponders. Four patients of each group were ACPA-positive. Mean total dose of infliximab for the responders and nonresponders were 8,367 and 8,257 mg, respectively. The control group comprised six healthy subjects with no report of any autoimmune disease or the use of any pharmacological drug. Blood samples from healthy donors were also obtained from the Partners Multiple Sclerosis Center at Brigham and Women’s Hospital under Institutional Review Board (IRB) Protocol 1999P010435 for cell surface antigen and intracellular cytokine-staining assays and in vitro Th17 cell differentiation analysis.
were isolated from total CD4+ T cells with the EasySep Human Naive CD4+ T Cell Isolation Kit (StemCell Technologies, catalog number 19555) following manufacturer’s instruction. CCRR6+ T cells were isolated from PBMC by negative selection using the EasySep Human CD4+CXCR3 CD20, CD36, CD56, CD66b, CD123, TCR Enrichment Cocktail, which removes cells expressing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγδ, glycophorin A, CD45RA, and CXCR3 by negative selection. Then CCRR6+ cells were selected using EasySep Human CCR6 Positive Selection Cocktail. Resulting CD4+CCCR6+CXCR3+ T cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with heat-inactivated 10% fetal calf serum and seeded in a U-bottom 96-well plate at 1–2 × 10^5 cells/100 μL/well. Equal volume of medium with or without PMA (60 ng/ml) and ionomycin (1 μg/mL) was added to each well. Cells were incubated at 37 °C, 5% CO2 for 4 h. Cells were then pelleted and lysed in 100 μL of Buffer RLT Plus containing 1 μL of β-mercaptoethanol (Qiagen, catalog number 1053939) following manufacturer’s instruction. Cell lysates were stored at –80 °C for later gene expression analysis.

**Cell Surface Antigen and Intracellular Cytokine-Staining Assays.** Total memory CD4+ T cells were isolated from PBMC by negative selection using the EasySep Human memory CD4+ T Cell Enrichment Kit (StemCell Technologies,
catalog number 19157) following manufacturer's instruction, and CCR6+ T cells were isolated as described in Cell Isolation and Stimulation. For cell surface lymphocytic staining, isolated cells were seeded into a U-bottom 96-well plate (up to 1 × 10^6 cells per well) and stained with anti-CD3 Brilliant Violet 605 (clone: OKT3, Biologend), anti-CD4-Pacific blue (clone: RPA-T4, BD Biosciences), anti-CD8-Fluor 700 (clone: HIT8a, Biologend), anti-CD19-FITC (clone: HIB19, Biologend), anti-CCR4-PE (clone: 1G12, BD Biosciences), anti-CCR6-Alexa Fluor 647 (clone: G034E3, Biologend), and anti-CXCR3-PerCP/Cy5.5 (clone: G025H7, Biologend). Cell surface antigen staining on CCR6+ lymphocytes was accessed with FlowJo. For intracellular cytokine staining, assays were carried out with staining buffers and antibodies from BD Biosciences as described before (19). Briefly, isolated cells were seeded into a U-bottom 96-well plate (up to 1 × 10^6 cells per well) and stimulated with PMA (100 ng/mL) and ionomycin (1 μg/mL) in the presence of GolgiStop (catalog number 554724) for 4 h. Cells were then fixed with BD Cytofix/Cytoperm buffer (catalog number 554723) following manufacturer’s instruction. The following fluorophore-conjugated antibodies from BD Biosciences were used for intracellular cytokine staining and the other for isotype control staining.

In Vitro Knockdown with shortRNA Lentiviruses in Th17 Cells. On day 0, naïve CD4+ T cells (30,000 cells) were cultured in a 96-well plate in X-VIVO 15 Serum-free Hematopoietic Cell Medium (Lonza Pharma & Biology, cat. number 01-4180) in the presence of STEMCELL ImmunoCult Human CD3/CD28 (SterCell Technologies, catalog number 10971). At 24 h, a lentiviral vector carrying a USF2-targeting shortRNA (GeneCoopelia, LPP-CS-HSH11132-shM03-01-100) was added to the culture at multiplicity of infection of 5 to knock down the expression of USF2. A lentiviral vector carrying a scramble shortRNA (GeneCoopelia, LPP-CS-HSH1001-shM03-300) was used as a control. The lentiviral vectors also expressed the enhanced green fluorescent protein (eGFP) reporter gene and puromycin resistant gene. At 48 h, cells were subjected to Th17 polarization condition: IL-1β (R&D, #201-LB005) at 0.5 μg/mL, IL-6 (R&D, #206-IL-010) at 25 ng/mL, IL-23 (R&D, #1290-IL-010) at 25 ng/mL, TGF-β (R&D, #240-B02) at 5 ng/mL, anti-IFN-γ (Clone B27) (BD Biosciences, #554698) at 1 μg/mL, and anti-IL-4 (Clone 34019) (R&D, #MAB204-100) at 1 μg/mL. On day 4, cells were treated with puromycin (Fisher Scientific, #554698) at 10 μg/mL. Live cells were sorted by a BD FACSAria sorter for RNA isolation.

Low-input Quantitative Real-Time PCR. Total RNA in cells (PBMC, CD4+ T cells, Th17 cells, and CCR6+ T cells) was isolated with the Qiagen RNeasy Plus Mini Kit (catalog number 74234). Complementary DNA (cDNA) was synthesized with the SuperScript VILO master mix (catalog number 11755050) and pre-amplified for 14 cycles with the TaqMan preAmp master mix (catalog number 4391128) following the manufacturer’s instruction. qPCR analysis was run and analyzed with the Viia 7 Real-Time PCR System (Life Technologies) using the TaqMan fast universal PCR master mix 2x (catalog number 432042) and qPCR primers (SI Appendix, Table S5) purchased from ThermoFisher Scientific. The comparative threshold cycle method and an internal control (β2m) were used for normalization of the target genes. Relative expression was calculated as ΔCt = Ctgene of interest − Ctβ2m, ΔΔCt = ΔCt sample of interest − mean of ΔCt healthy controls, the relative change of gene expression between the expression level of sample of interest and the mean expression level of healthy controls was given by this formula: (2^−ΔΔCt) × 10. All qPCR reactions were performed in duplicate.

nCounter Analysis of Gene Expression. As previously described, we designed a nanoString CodeSet HuT717 that constitutes a 418-gene expression detection panel specific for human T cell activation and differentiation (19). Cell lysates prepared as described above from stimulated and unstimulated isolated CCR6CD4+ cells were subjected to the nCounter Gene Expression Analysis using the CodeSet HuT717 according to the protocol provided by the manufacturer (NanoString Technologies).

Data Analysis. Similar to the approaches we described previously (19), nCounter gene expression data were normalized for code count using the geometric mean, for background using the mean, and for sample content using the geometric mean of housekeeping genes (B2M, RPL3, and beta actin) with R (version 3.2.0) and NanoStringNorm (version 1.2.1). Mean ± 2 SDs of detected expression values of negative controls of the CodeSet HuT717 was used as the cutoff to select for expressed genes, which have the minimum expression value across all samples in an unbiased manner above the cutoff value. Using this criterion, 397 of the 415 genes (excluding the three housekeeping genes) were selected as expressed genes and subjected to further analyses. Gene expression heat maps were generated with GENE-E (https://software.broadinstitute.org/GENE-E) using color-value as the z-scores of a data point. PCA plots were generated using R, factoextra (v1.0.5), and ggplot2 (v2.2.1). As described previously (19), mouse gene expression data were downloaded from the Gene Expression Omnibus (GEO) (GSE37320) (19) and normalized with Robust Multi-array Average (RMA) (67) and ComBat (68) in GenePattern (https://www.genepattern.org/), and genes with multiple probes were collapsed to unique genes by selecting the probe with the highest average expression across all samples. GSEA was done in GenePattern using default settings (weighted scoring scheme, SignaInsNoise (1,000 permutations) (69–71) to test the enrichment of human signatures in the mouse expression profiles. Storey’s q-value is used to control the false discovery rate. The IPA canonical pathway analysis and upstream regulator analysis were performed for the differentially expressed genes (using corresponding fold changes and P values) to identify key upstream regulatory molecules. Canonical pathways and upstream regulators with z-scores ≥ 2 and z-scores ≤ −2 were defined as activator and inhibitor mechanisms, respectively. IPA was also used to generate the network diagram. In addition, Enrichr (https://maayanlab.cloud/Enrichr/) was also utilized to conduct transcriptional and pathway analysis, in which the P value is computed from the Fisher exact test; the adjusted P value is a rank-based ranking derived from running the Fisher exact test for many random gene sets to compute a mean rank and SD from the expected rank for each term in the gene set library. The z-score is defined as the deviation from the expected rank, and the combined score is calculated by log of the P value multiplied by the z-score.

Statistical Analysis. Paired Student’s t test was performed to compare the T cell subset frequencies. Unpaired Student’s t test was performed to compare differential gene expression between responders vs. nonresponders, responders vs. HC, and nonresponders vs. HC, respectively, with Excel. Two-tailed P values < 0.05 were considered statistically significant. Statistical analysis was performed with Prism 7 and GraphPad Software, and one-way ANOVA was performed with R statistical software (version 3.2.0) to compare the differential gene expression among healthy controls and responding and nonresponding patients with RA. Genes with P value < 0.05 were selected for PCA (Fig. 2 C and D).

Data Availability. The main data supporting the findings of this study are available within the article and SI Appendix.
8. X. Chen et al., 991–999 (2012).