Dysregulation of proinflammatory versus anti-inflammatory human T_H17 cell functionalities in the autoinflammatory Schnitzler syndrome

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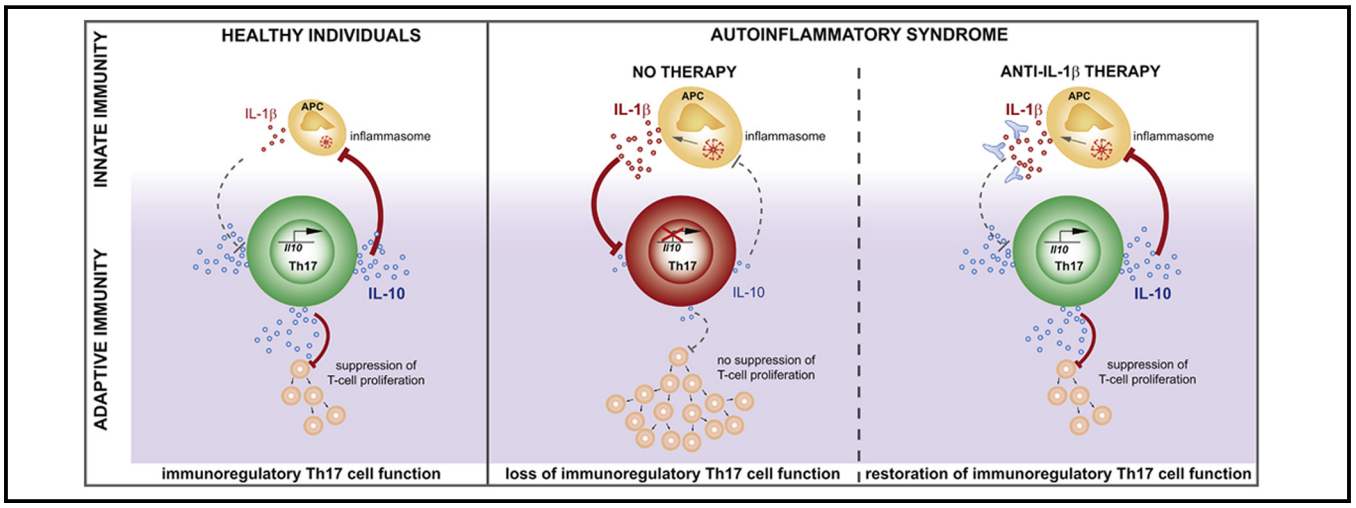
GRAPHICAL ABSTRACT



Background: T_H17 cells have so far been considered to be crucial mediators of autoimmune inflammation. Two distinct types of T_H17 cells have been described recently, which differed in their polarization requirement for IL-1β and in their cytokine repertoire. Whether these distinct T_H17 phenotypes translate into distinct T_H17 cell functions with implications for human health or disease has not been addressed yet.

Objective: We hypothesized the existence of proinflammatory and anti-inflammatory human T_H17 cell functions based on the differential expression of IL-10, which is regulated by IL-1β. Considering the crucial role of IL-1β in the pathogenesis of autoinflammatory syndromes, we hypothesized that IL-1β mediates the loss of anti-inflammatory T_H17 cell functionalities in patients with Schnitzler syndrome, an autoinflammatory disease.

Methods: To assess proinflammatory versus anti-inflammatory T_H17 cell functions, we performed suppression assays and tested the effects of IL-1β dependent and independent T_H17 subsets on modulating proinflammatory cytokine secretion by monocytes. Patients with Schnitzler syndrome were analyzed for changes in T_H17 cell functions before and during therapy with IL-1β–blocking drugs.

Results: Both T_H17 cell subsets differ in their ability to suppress T-cell proliferation and their ability to modulate proinflammatory cytokine production by antigen-presenting cells because of their differential IL-10 expression properties. In patients with Schnitzler syndrome, systemic overproduction of IL-1β translates into a profound loss of anti-inflammatory T_H17 cell functionalities, which can be reversed by anti–IL-1β treatment.

Conclusion: IL-1β signaling determines the differential expression pattern of IL-10, which is necessary and sufficient to induce proinflammatory versus anti-inflammatory T_H17 cell functions. Our data introduce T_H17 cell subsets as novel players in autoinflammation and thus novel therapeutic targets in

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autoinflammatory syndromes including other IL-1β mediated diseases. This demonstrates for the first time alterations in the adaptive immune system in patients with autoinflammatory syndromes. (J Allergy Clin Immunol 2016;138:1161-9.)

Key words: *Autoinflammation, Schnitzler syndrome, T_H17 cells, IL-10, IL-1β*

Abbreviations used
FACS: Fluorescence-activated cell sorting
IL-1Ra: IL-1 receptor antagonist
Treg: Regulatory T

T_H17 cells have been recognized as critical players in the pathogenesis of autoimmune diseases and in the clearance of extracellular pathogens and fungi.[1,2] In mice terminal differentiation of T_H17 cells is dependent on IL-23, which abrogates the induction of IL-10 and promotes IFN-γ coexpression in IL-17–producing T helper cells. This confers pathogenicity to T_H17 cells, as evidenced most strikingly in the EAE mouse model of multiple sclerosis.[3] In human subjects 2 types of stable T_H17 cells have recently been identified *in vivo*.[4] They differ in their property to coexpress either IL-10 or IFN-γ and in their microbial antigen specificities. These distinct phenotypes are generated with differential requirements for IL-1β during the polarization phase. In humans IL-1β, but not IL-23, strongly suppresses IL-10 and promotes IFN-γ/T-bet induction.[4]

Despite this differential cytokine coexpression pattern, it has not been demonstrated so far whether these distinct human T_H17 cell phenotypes indeed translate into different functionalities. Although both subsets continue to express proinflammatory IL-17, the question of whether anti-inflammatory IL-10 might confer a dominant immunosuppressive functionality on T_H17 cells is warranted. This would necessitate a rethinking about the general concept of T_H17 cells being major mediators of inflammatory tissue destruction.

Autoinflammatory syndromes are characterized by overproduction of IL-1β caused by mutations in the inflammasome, a large intracellular multiprotein complex that leads to maturation of IL-1β and IL-18.[5] Due to the absence of inflammasome expression in cells of the adaptive immune system, autoinflammation has been considered to be caused solely by abrogations in innate immunity, although this could potentially also translate into downstream alterations of adaptive immune functions. Schnitzler syndrome belongs to this group of very rare autoinflammatory syndromes. It presents with a chronic urticarial rash, intermittent fever, arthralgia or arthritis, hepatomegaly and/or splenomegaly, lymphadenopathy, leukocytosis, and an increased erythrocyte sedimentation rate.[6,7] We recently demonstrated 2 variant cases with somatic mosaicism of *NLRP3* mutations in the myeloid lineage, leading to spontaneous IL-1β secretion by PBMCs.[8] Furthermore, increased IL-1β secretion by LPS-stimulated PBMCs was reported.[9] Hence, apart from the monoclonal IgM gammopathy, the immunologic significance of which is unknown, and IL-1β overproduction, no immune-related abnormalities have been detected in patients with Schnitzler syndrome so far.[6]

Because IL-1β is the critical switch factor that determines whether T_H17 cells can coexpress IL-10,[4] Schnitzler syndrome represents an "experiment of nature" in which alterations in adaptive immunity caused by systemic IL-1β overproduction can be studied *in vivo* in human subjects. In addition, immunologic perturbations, such as systemic *in vivo* inhibition of IL-1β by targeted therapies with IL-1 receptor antagonist (IL-1Ra; anakinra) and IL-1β–neutralizing antibodies (canakinumab and gevokizumab), can be performed *in vivo* in this disease setting.

This revealed the existence of 2 functionally distinct subsets of human T_H17 cells, which can be categorized into proinflammatory versus anti-inflammatory subsets because of the dominant role of IL-10 and despite the heterogeneous cytokine repertoire of both T_H17 cell subsets. We also demonstrate a loss of anti-inflammatory T_H17 cells in patients with Schnitzler syndrome, which could be restored to physiological levels upon therapeutic IL-1β inhibition. This could have implications for disease pathogenesis and future therapeutic strategies.

METHODS

Cell purification and sorting

$CD14^+$ monocytes and $CD4^+$ T cells were isolated from PBMCs after density gradient sedimentation with Ficoll-Paque Plus (GE Healthcare, Fairfield, Conn) by means of positive selection with CD14- or CD4-specific microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). T helper cell subsets were sorted to greater than 97% purity as follows: T_H1 subset, $CCR6^-CCR4^-CXCR3^+CD45RA^-CD25^-CD8^-$; T_H2 subset, $CCR6^-CCR4^+CXCR3^-CD45RA^-CD25^-CD8^-$; and T_H17 subset, $CCR6^+CCR4^+CXCR3^-CD45RA^-CD25^-CD8^-$. The following antibodies were used for fluorescence-activated cell sorting (FACS): phycoerythrin-conjugated anti-CCR6 (11A9; BD Biosciences, San Jose, Calif), phycoerythrin-indotricarbocyanine–conjugated anti-CCR4 (1G1; BD Biosciences), allophycocyanin-conjugated anti-CXCR3 (1C6/CXCR3; BD Biosciences), phycoerythrin-Cy5–conjugated anti-CD45RA (HI100, BD Biosciences), R phycoerythrin cyanin 5.1–conjugated anti-CD8 (B9.11; Coulter Immunotech, Krefeld, Germany), and fluorescein isothiocyanate–conjugated anti-CD25 (B1.49.9; Immunotech). Naive T cells were isolated as $CD45RA^+CD45RO^-CCR7^+CD25^-CD8^-$ to a purity of greater than 99% after staining with the aforementioned antibodies against CD45RA, CD25, and CD8, as well as fluorescein isothiocyanate–conjugated CD45RO (UCHL1; Immunotech) and anti-CCR7 (150503; R&D Systems, Minneapolis, Minn), followed by staining with biotinylated anti-IgG_{2a} (1080-08; SouthernBiotech, Birmingham, Ala) and streptavidin–Pacific blue (Molecular Probes, Invitrogen, Eugene, Ore). Permission for experiments with human primary cells was obtained from the ethics committee of the Charité-Universitätsmedizin Berlin (EA1/221/11), the ethics committee of the University of Wuerzburg (protocol no. 239/10), and the Radboud University Medical Center, Nijmegen, with approval by the local medical ethics committee, as well as from the ethics committee of the Technical University of Munich (195/15s). Samples from patients with Schnitzler syndrome from the NCT01390350[10] cohort were obtained before and at different time points after treatment with canakinumab during a proof-of-principle immunologic study at the Charité-Universitätsmedizin Berlin. Patients were eligible for enrollment in the study if they had active Schnitzler syndrome. All patients selected for this study responded to treatment with complete remission, as defined by physician's global assessment on overall autoinflammatory disease activity. For more information on clinical trial details, we refer to NCT01390350 and Krause et al.[10]

Samples from patients with Schnitzler syndrome after treatment with gevokizumab were obtained from the Nijmegen Center for Immunodeficiency and Autoinflammation. Clinical trial information is deposited in the EU Clinical Trials Register (EudraCT no. 2013-002562-39). PBMCs of patients with systemic juvenile idiopathic arthritis (Still disease) who were naive for any biologic treatment were obtained from the Laboratory of Pediatric Rheumatology and Special Immunology, University Children's Hospital, University of Wuerzburg and the German Registry for Autoinflammatory Diseases (AID-Net).

Cell sorting was performed with a FACSAria III or MoFlo (Beckman Coulter, Brea, Calif). Flow cytometry was performed with a CytoFLEX (Beckman Coulter), FACSCanto (BD Biosciences), and MACSQuant (Miltenyi Biotec). Flow cytometric data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Cell culture

Cells were cultured in RPMI-1640 medium supplemented with 2 mmol/L glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate, penicillin (50 U/mL), streptomycin (50 μg/mL; all from Invitrogen, Carlsbad, Calif), and 5% (vol/vol) human serum (Charité Blood Center). T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), according to standard protocols. Monocytes were cultured for 24 hours with or without LPS (100 ng/mL; Sigma-Aldrich, St Louis, Mo).

Suppression assays

Naive $CD45RA^+$ CFSE-labeled $CD4^+$ T cells were cocultured as responder T cells with equal or titrated numbers of different T helper cell subsets in the presence of equal numbers of irradiated anti-CD3–coated (OKT3) PBMCs (28 Gy) as feeder cells. In some experiments naive but also T helper cell subsets were used as responder cells, where indicated. CFSE dilution was analyzed on day 4 by FACS. For IL-10 neutralization, purified rat anti-human IL-10 (clone JES3-19F1, isotype control rat IgG_{2a}) was used at a concentration of 10 μg/mL. For suppression assays with material from patients, we cocultured the patients' T_H17 cells with healthy allogeneic naive T cells, which allowed us to assess the suppressive properties of the patients' T_H17 cells, irrespective of any potential alterations in the patients' naive T-cell (responder cell) proliferation capacity.

Cytokine analyses

For intracellular cytokine staining, T cells were restimulated for 5 hours with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A (all from Sigma-Aldrich) for the final 2.5 hours of culture. Monocytes were stimulated for 40 hours with LPS (100 ng/mL, Sigma) in the presence of brefeldin A and the absence of phorbol 12-myristate 13-acetate and ionomycin. Cells were fixed and made permeable with Cytofix/Cytoperm, according to the manufacturer's instructions (BD Biosciences). Cells were stained with phycoerythrin- or allophycocyanin-conjugated anti–IL-10 (both JES3-19F1; BD Biosciences) and Alexa Fluor 647– or fluorescein isothiocyanate–conjugated anti–IL-17 (both eBIO64-DEC17; eBioscience, San Diego, Calif), PE/Cy7-conjugated TNF-α (BioLegend, San Diego, Calif), and phycoerythrin- or allophycocyanin-conjugated anti–GM-CSF (R&D Systems). Flow cytometric data were analyzed with FlowJo software. Cytokines in T-cell culture supernatants were measured by using ELISA according to a standard protocol after restimulation of cultured T cells (5×10^4 cells in 200 μL) with phorbol-12-13-dibutyrate (50 nmol/L, Sigma-Aldrich) and plate-bound anti-CD3 (1 μg/mL, TR66) for 8 hours. Data were analyzed by the Softmax program. IL-1β and TNF-α secretion by monocytes was measured by using the cytometric bead array, according to the manufacturer's instructions (BD Biosciences), as well as by ELISA (DuoSet ELISA Kits; R&D Systems).

Statistics

The Student *t* test was used to test the statistical significance of the results. *P* values of .05 or less were considered statistically significant.

RESULTS

Human T_H17 cells have IL-10–dependent suppressive properties

T_H17 cells have a heterogeneous cytokine profile that goes beyond production of their lineage identifying cytokines IL-17 and IL-22.[1] To investigate whether human T_H17 cells have anti-inflammatory properties, we performed T-cell suppression assays, which are classical surrogate assays to demonstrate regulatory activities of regulatory T (Treg) cells in both mice and human subjects. T_H17 cells from healthy control subjects were sorted according to the differential expression of CCR6, CXCR3, and CCR4, which we have previously shown to highly enrich for the T_H17 cell subset at the exclusion of T_H1 and T_H2 cells.[4,11] To further exclude potentially contaminating Treg cells, we performed negative sorting for CD25 expression. Surprisingly, T_H17 cells suppressed the proliferation of naive responder T helper cells in a dose-dependent manner (Fig 1, *A*). We next addressed the mechanism of T_H17 cell–mediated responder T-cell suppression. IL-10 is a classical anti-inflammatory cytokine that can be produced by the $CCR6^+CXCR3^-CCR4^+$ T_H17 cell subset.[4] Compared with the T_H1 and T_H2 cell subsets, T_H17 cells have the highest IL-10 coexpression at the single-cell level after polyclonal stimulation with plate-bound CD3 and CD28 mAbs (Fig 1, *B* and *C*).

For this reason, we analyzed whether IL-10 mediated the suppression of responder T cells. Blocking IL-10 with neutralizing antibodies was indeed sufficient to reverse responder T-cell suppression by T_H17 cells to levels of responder cell proliferation seen in the absence of suppressor cells (Fig 1, *D*). This demonstrates that their ability to produce IL-10 provides T_H17 cells with net immunosuppressive functions. This was surprising considering that they continue to produce proinflammatory cytokines, such as GM-CSF, which has been reported to confer pathogenic functions to T_H17 cells in the EAE mouse model[12,13] and correlates with multiple sclerosis in human subjects (see Fig E1 in this article's Online Repository at www.jacionline.org).[14] Therefore IL-10 exerts a dominant immunosuppressive functionality on human T_H17 cells despite coexpression of other proinflammatory cytokines, such as IL-17 and GM-CSF.

We have also dissected the ability of T_H17–$IL\text{-}10^+$ cells to differentially suppress distinct T helper cell subsets in addition to naive T helper cells. Our data demonstrate that T_H17 cells can suppress the proliferation of not only naive responder cells but also effector T helper cell subsets of different lineages with variable efficiency (see Fig E2 in this article's Online Repository at www.jacionline.org).

Together, these data demonstrate IL-10–dependent suppressive properties of human T_H17 cells in an *in vitro* suppression assay.

Activation increases regulatory properties of human T_H17 cells

Human T_H17 cells have previously been demonstrated to upregulate IL-10 expression upon T-cell receptor stimulation.[4] To test whether T_H17 cell activation translates into more potent suppressive properties, we preactivated human T_H17 cells for 48 hours with CD3 and CD28 mAbs and assessed responder T-cell suppression (Fig 2). For comparison of regulatory strengths, we also performed suppression assays with professional $CD25^{high}CD127^{low}$ Treg cells, which expressed greater than 90% forkhead box protein 3 (see Fig E3 in this article's Online Repository at www.jacionline.org). In cultures with T_H17 cells that were not preactivated but only received weak activation stimuli from allogeneic anti-CD3–coated feeder cells during the coculture period, 29.6% ± 4.5% (mean ± SEM) of responder cells proliferated. This low stimulation strength was consistent with low IL-10 expression levels by nonpreactivated T_H17 cells[4] and correlated with low suppressive function (Fig 2, *B*).

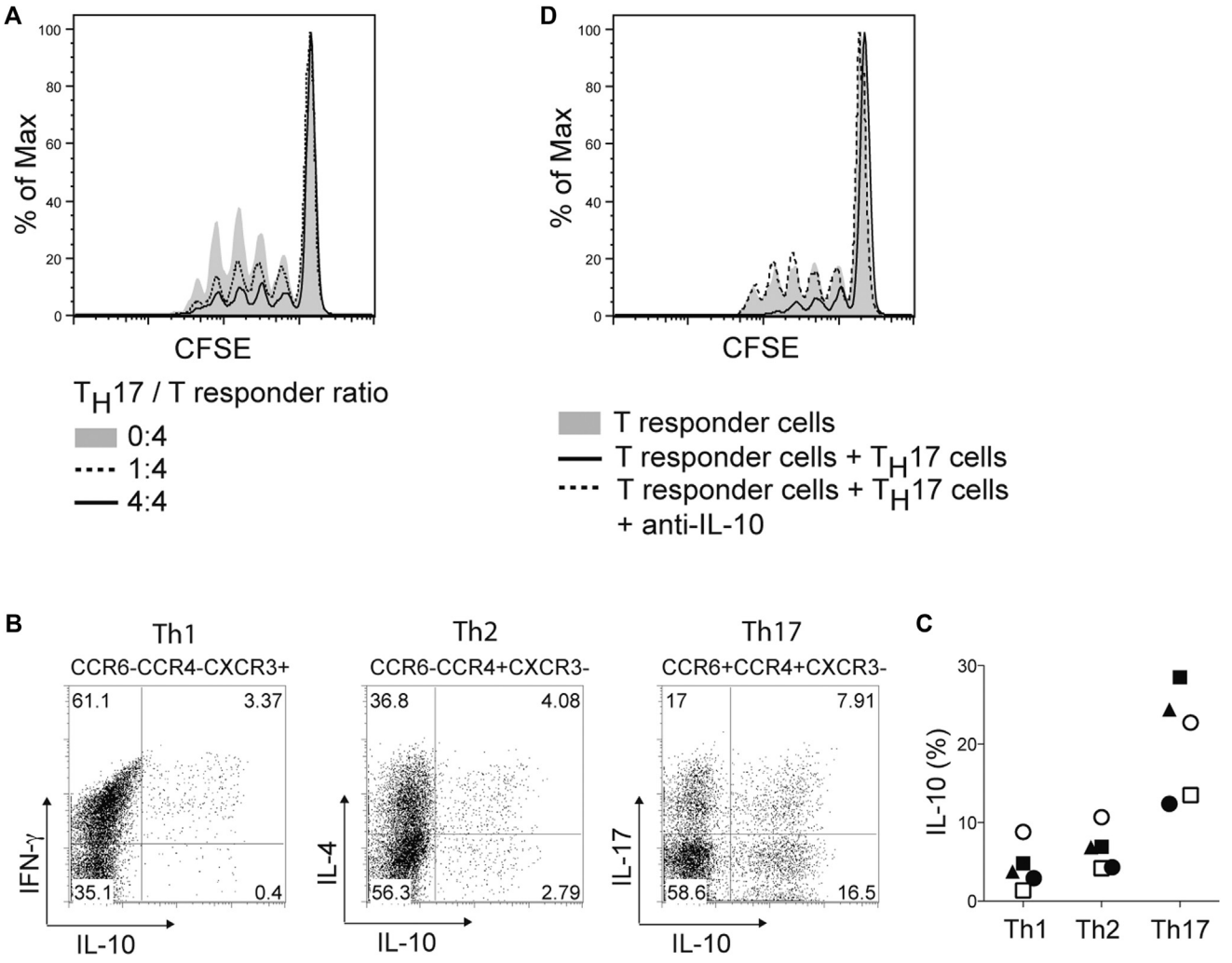

FIG 1. Human T_H17 cells have IL-10–dependent regulatory functions. **A,** T_H17 cells were isolated as $CXCR3^-CCR4^+CCR6^+CD25^-$ T helper cells and cocultured at different ratios with naive CFSE-labeled responder T cells in the presence of feeder cells for 3 days. Data are representative of 3 individual experiments. **B** and **C,** Intracellular staining and flow cytometry of human T helper cell subsets on day 5 after 48 hours of stimulation with CD3 and CD28 mAbs. T helper cell subsets were isolated according to the differential expression of CXCR3, CCR4, and CCR6, as indicated. Different symbols indicate individual donors. **D,** The suppression assay was performed as in Fig 1, *A*, with equal numbers of responder and T_H17 cells with or without addition of anti–IL-10 (10 μg/mL).

In cultures with Treg cells, only 3.2% ± 1.0% (mean ± SEM) of responder cells proliferated compared with 53% ± 5.2% in the absence of suppressor cells. As expected, polyclonal preactivation with plate-bound CD3 and CD28 antibodies induced IL-10 upregulation in T_H17 cells (not preactivated, 2.0% ± 0.8%; preactivated, 13.8% ± 2.4%). This correlated with an increase in their suppressive function (responder cell proliferation, 2.7% ± 0.6%). Interestingly, the suppressive strength of activated IL-10–producing T_H17 cells was even comparable with that of Treg cells (no significant difference, $P = .7$; Fig 2), which are specialized in immunosuppression by multiple mechanisms.[15]

Two types of human T_H17 cells that differ in regulatory properties

Human T_H17 cell subsets with distinct antigen specificities and differential priming requirements for IL-1β, which determines either IL-10 or IFN-γ coexpression patterns, have been described recently.[4] Whether this phenotypic diversity within the T_H17 cell subset translates into functional differences has not been analyzed yet. We hypothesized that there are differential suppressive properties of $IL\text{-}10^+$ versus $IL\text{-}10^-$ T_H17 cells. To test this, $CCR6^+CXCR3^-CCR4^+$ T_H17 cells, which we had demonstrated to coproduce IL-10,[4] were restimulated with IL-1β for 48 hours. This completely suppressed the ability of T_H17 cells to express IL-10, which is in accordance with published data (see Fig E4 in this article's Online Repository at www.jacionline.org).[4] IL-1β–treated and untreated T_H17 cells, which differed in their ability to coexpress IL-10, were then used for suppression assays. In accordance with our hypothesis, we observed that both T_H17 cell subsets differed in their suppressive functions. With $IL\text{-}10^-$ T_H17 cells, responder cell proliferation amounted to 83.5% of the control responder T-cell proliferation in the absence of suppressive T cells. This did not differ statistically from control

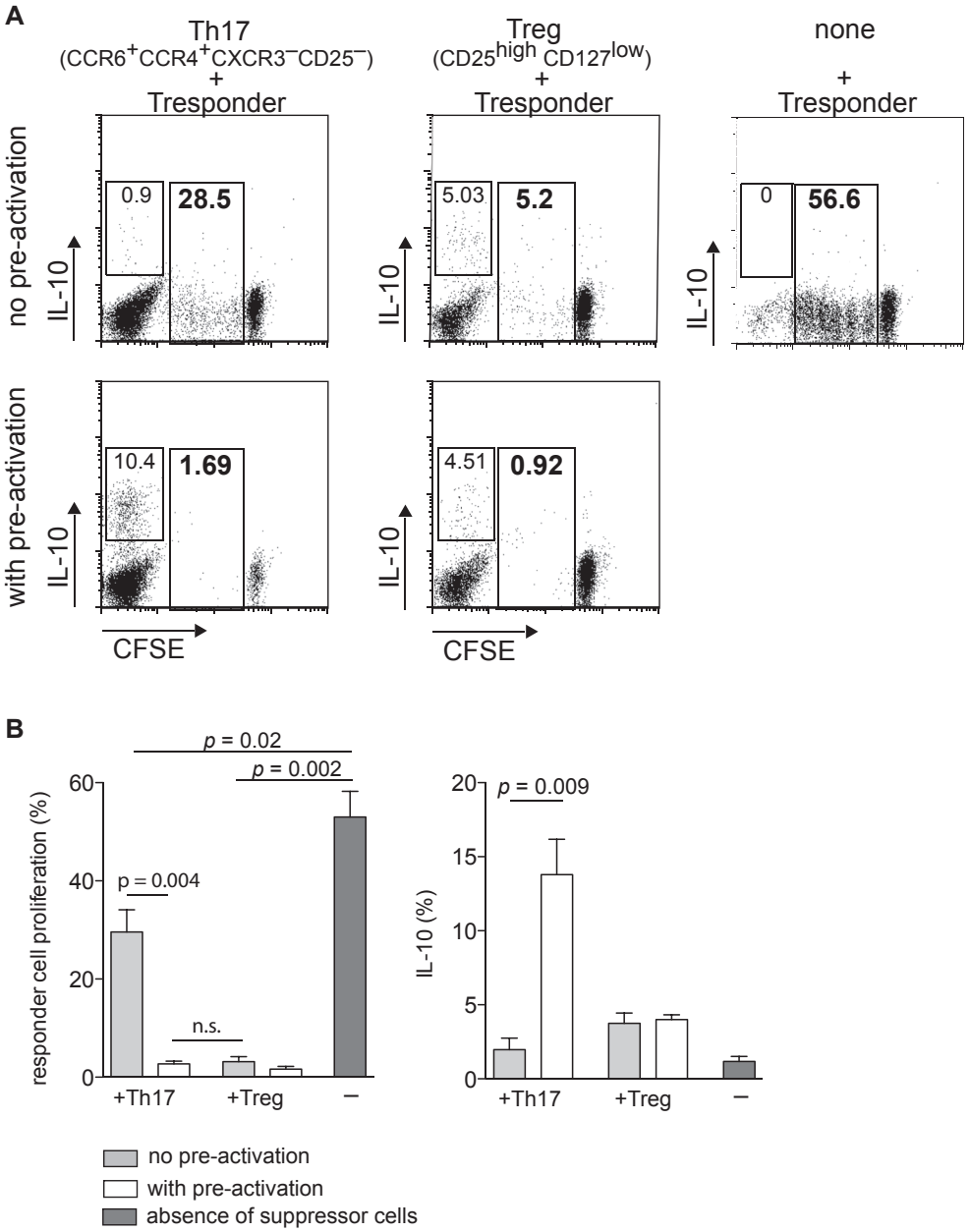

FIG 2. Regulatory properties of human T_H17 cells increase after T-cell receptor activation. **A** and **B,** Suppression assays with T_H17 and control Treg cells with and without prior stimulation with CD3 and CD28 mAbs for 48 hours were performed. *Numbers in vertical gates* indicate percentages of IL-10^+ cells (small regular font) and percentages of proliferating cells (large boldface font) among all viable cells. Dot plot presentation was chosen to simultaneously demonstrate proliferation by means of CFSE dilution, as well as cytokine expression of CFSE-unlabeled T_H17 and Treg cells. Data are representative of 3 experiments (Fig 2, *A*) and cumulative data (Fig 2, *B*). *Error bars* indicate means ± SEMs (n = 3). *n.s.,* Not significant.

responder T-cell proliferation ($P = .2$), whereas with IL-10^+ T_H17 cells, responder T-cell proliferation was strongly impaired (>3-fold reduction, Fig 3). This demonstrated that IL-1β acts as a critical switch factor for determining proinflammatory versus anti-inflammatory functionalities of human T_H17 cells.

Two types of T_H17 cells that differ in their property to modulate innate cytokine secretion

Suppression assays are considered powerful surrogates for determining the regulatory properties of T cells *in vitro*. Even though they have clearly demonstrated the suppressive properties of Treg cells previously, it has been questioned whether *in vivo* properties of T cells with regulatory functions are reflected by this *in vitro* assay with high fidelity.[16] To corroborate our finding of differential regulatory properties of T_H17–IL-10^+ and T_H17–IL-10^- cells, we also used another experimental readout for regulatory functions. We therefore tested the ability of both T_H17 types to downregulate production of proinflammatory cytokines, in particular IL-1β, by antigen-presenting cells, such as monocytes. $CD14^+$ monocytes were

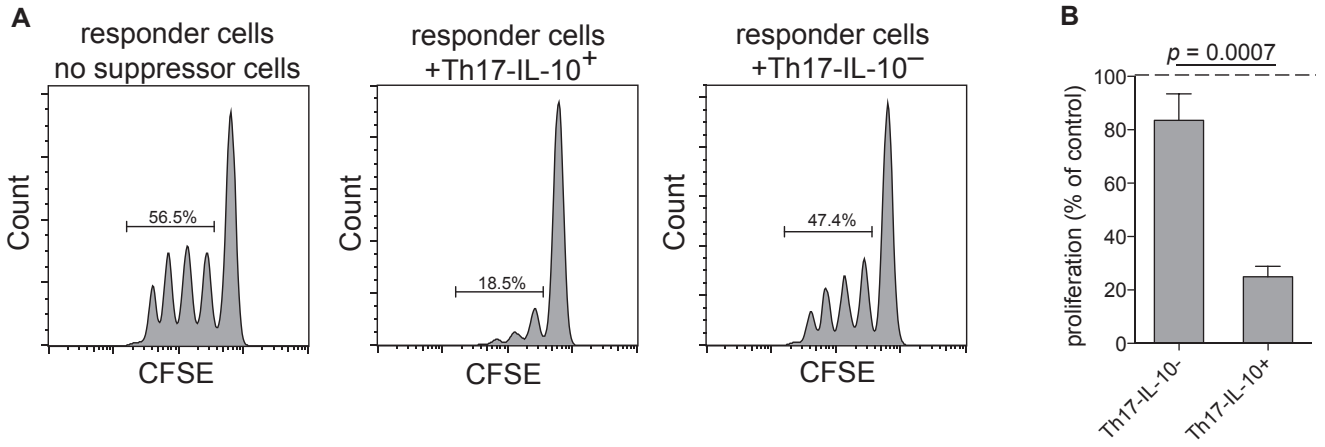

FIG 3. Two types of T_H17 cells that differ in their suppressive properties. **A** and **B,** Th17 cells were isolated as $CXCR3^-CCR4^+CCR6^+$ T cells and restimulated in the presence (T_H17–IL-10^-) or absence (T_H17–IL-10^+) of IL-1β (10 ng/mL) for 48 hours with plate-bound CD3 and CD28 mAbs. T_H17–IL-10^- and T_H17–IL-10^+ cells were then used for suppression assays with naive CFSE-labeled T helper cells from the same donors. CFSE dilution was assessed on day 4 by using FACS. *Bars* indicate percentages of proliferating responder cells among all viable cells. Data are representative of 6 experiments (Fig 3, *A*) or cumulative data (Fig 3, *B*). *Error bars* indicate means ± SEMs (n = 6). Due to variability of responder T-cell proliferation in different experiments, the proliferating responder cell fraction in the absence of suppressor cells (control condition) is set to 100%. Shown is the percentage of proliferating responder cells in the presence of suppressor cells in relation to the control condition (Fig 3, *B*). No significant difference in proliferation of responder cells in the absence of suppressor cells (control) and in the presence of T_H17–IL-10^- was detected ($P = .18$).

stimulated with LPS, which is sufficient for induction of IL-1β secretion in human subjects in accordance with published data.[17] They were cocultured with 48-hour preactivated T_H1, T_H2, and T_H17 cells, which were isolated according to their differential expression of CXCR3, CCR4, and CCR6. T_H17 cells efficiently suppressed the secretion of bioactive IL-1β by monocytes (Fig 4, *A*). Interestingly, the T_H17 cell subset was the most efficient T helper cell subset for innate IL-1β suppression compared with the T_H1 and T_H2 cell subsets. This correlated with its ability to produce the highest levels of IL-10 upon activation (Fig 4, *B*). This suppressive property was not only restricted to IL-1β secretion. T_H17 cells, but not T_H1 and T_H2 cells, also significantly suppressed the secretion of other proinflammatory cytokines, such as TNF-α, by monocytes in response to LPS (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org), although the frequency of TNF-α^+ monocytes was not altered after coculture with different T helper cell subsets (see Fig E5, *B*).

To test whether the 2 distinct T_H17 cell subsets differed in their ability to suppress proinflammatory cytokine secretion by monocytes, T_H17 cells that were preactivated in the presence (T_H17–IL-10^-) or absence (T_H17–IL-10^+) of IL-1β were cocultured with LPS-stimulated monocytes (Fig 4, *C*). Only T_H17–IL-10^+ cells suppressed IL-1β secretion. This was dependent on their IL-10 secretion because addition of IL-10–neutralizing antibodies to the coculture abrogated their ability to downregulate IL-1β secretion. Exogenous addition of recombinant IL-10, on the other hand, resulted in reduced IL-1β secretion, even in coculture with T_H17–IL-10^- cells, highlighting a role of soluble IL-10 as a potent suppressor of IL-1β secretion. Interestingly, our control condition with LPS-stimulated monocytes alone demonstrated that addition of IL-10–neutralizing antibodies also increases IL-1β secretion in the absence of T cells, indicating autocrine IL-10 secretion by monocytes as a negative feedback mechanism for the proinflammatory IL-1β production.

Together, these results demonstrate a novel property of T_H17 cells. They can downregulate the inflammatory properties of antigen-presenting cells, such as monocytes, by reducing secretion of proinflammatory cytokines, including IL-1β and TNF-α. In addition, we revealed that T_H17 cells differed in their ability to mediate this anti-inflammatory function depending on whether they belonged to the IL-10^+ or IL-10^- subset.

T_H17 cells from patients with Schnitzler syndrome have reduced regulatory properties, which are restored with IL-1β–targeting therapies

Autoinflammatory syndromes comprise a group of rare, mostly hereditary inflammatory disorders, which present with recurrent fevers, skin rashes, and arthritis.[18] Inflammasome defects lead to overproduction of IL-1, which mediates immunopathology. Because we had demonstrated that IL-1β suppressed IL-10 production by T_H17 cells,[4] we hypothesized that patients with autoinflammatory syndromes have reduced regulatory T_H17 cell functions and that IL-1β–blocking therapies, such as IL-1Ra (anakinra) or anti–IL-1β (canakinumab and gevokizumab), would restore regulatory properties in T_H17 cells. Therefore autoinflammatory syndromes provide the unique opportunity to study in an "experiment of nature" human regulatory T_H17 cell functions and their therapeutic perturbations *in vivo*.

We isolated T_H17 cells from healthy age-matched donors, as well as from patients with Schnitzler syndrome, an autoinflammatory syndrome,[19,20] and performed suppression assays with healthy memory responder T helper cells. Interestingly, T_H17 cells from patients with Schnitzler syndrome showed inferior suppressive functions compared with T_H17 cells from healthy donors in all experiments (Fig 5, *A*). This correlated with a

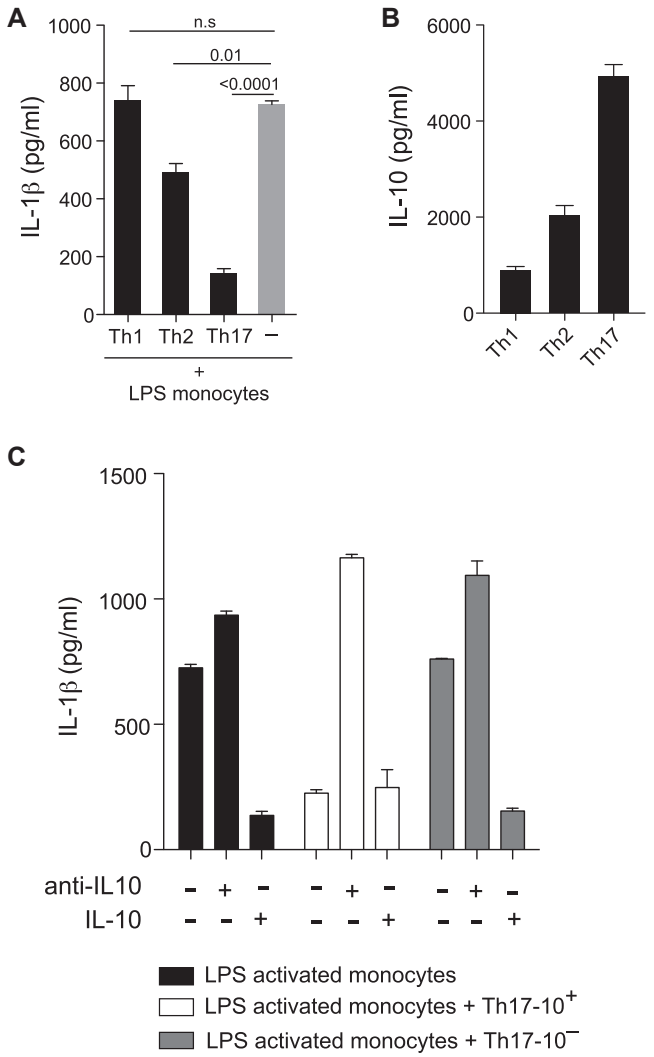

FIG 4. T_H17 cells suppress IL-1β secretion by monocytes through IL-10 production. **A,** Monocytes were stimulated with LPS for 48 hours and cocultured with T_H1, T_H2, and T_H17 cell subsets that have been isolated according to the differential expression of CXCR3, CCR4, and CCR6 and preactivated for 48 hours with CD3 and CD28 mAbs before coculture for 48 hours. IL-1β was analyzed by using a cytometric bead array. Data show means ± SEMs (n = 3). Numbers indicate *P* values (paired Student *t* test). **B,** T_H1, T_H2, and T_H17 cell subsets were isolated *ex vivo* as in Fig 4, *A*, and stimulated for 48 hours with plate-bound CD3 and CD28 mAb. IL-10 levels were measured by ELISA after restimulation of equal numbers of preactivated T cells with phorbol-12-13-dibutyrate and plate-bound anti-CD3 (1 μg/mL, TR66) for 8 hours. Data show mean ± SEMs (n = 3). **C,** Monocytes were stimulated as in Fig 4, *A*, in the presence or absence of T_H17–IL-10^+ or T_H17–IL-10^- cells that had been generated by restimulation of T_H17 cells ($CCR6^+CCR4^+CXCR3^-$ memory T cells) in the presence or absence of IL-1β, respectively. IL-10–blocking mAbs (10 μg/ml) or recombinant IL-10 (10 ng/mL) was added where indicated. Supernatant was analyzed after 48 hours by cytometric bead array. Data show mean ± SEMs (n = 3).

significantly reduced ability of T_H17 cells from patients with Schnitzler syndrome to produce IL-10 upon polyclonal restimulation (Fig 5, *B* and *C*). Reduced IL-10 expression levels were not only restricted to T_H17 cells from patients with Schnitzler syndrome but were also observed in T cells from patients with systemic juvenile idiopathic arthritis, another autoinflammatory disease, which is in line with their increased systemic IL-1β levels (see Fig E6 in this article's Online Repository at www.jacionline.org). IL-17 production was variable and did not show statistical differences between patients and healthy control donors (cumulative data not shown).

We next isolated T_H17 cells from patients with Schnitzler syndrome under treatment with IL-1β–blocking drugs. We hypothesized that therapeutic abrogation of IL-1β signaling in patients with Schnitzler syndrome would likewise restore regulatory functions of T helper cell subsets. Indeed, IL-10 expression was restored to baseline levels after treatment with the IL-1β–neutralizing antibody canakinumab within the same patients after 6 months of treatment (matched pairs). All patients had systemic inflammation, including recurrent fevers and arthralgias caused by IL-1β overproduction that subsided on the aforementioned IL-1β–blocking therapies, resulting in complete remission.[10]

To further corroborate this finding, we also analyzed IL-10 expression in T_H17 cells from patients with Schnitzler syndrome undergoing treatment with gevokizumab (n = 2), another IL-1β mAb, as well as anakinra, an IL-1Ra (n = 1), which amounts to a total of 6 patients with this very rare disease. We observed increased IL-10 expression levels compared with those in patients who did not receive any IL-1β–targeting treatment ($P = .05$; Fig 5, *C*). Although IL-10 expression was significantly upregulated with gevokizumab and anakinra, it did not amount to the same high levels seen in patients receiving canakinumab treatment. Nevertheless, in all of the patients analyzed, irrespective of the mode of IL-1β blockade, we observed increased IL-10 expression on IL-1β–blocking treatments (Fig 5, *B* and *C*). In addition, IL-1β–blocking therapy with canakinumab improved the regulatory properties of T_H17 cells in suppression assays (Fig 5, *D*). Together, these experiments demonstrate that patients with Schnitzler syndrome have T_H17 cells with reduced regulatory properties, which can be restored by IL-1β–blocking therapies.

DISCUSSION

T_H17 cells have thus far been considered critical players in autoimmune tissue inflammation, as well as in pathogen clearance. Recently, GM-CSF, but not IL-17, has been shown to be the crucial pathogenic T_H17 cell cytokine in the EAE mouse model for multiple sclerosis and to be also associated with human multiple sclerosis.[12,14] This demonstrates that the pathogenicity of T_H17 cells is determined by specific cytokine coexpression patterns rather than by IL-17 expression alone. This also suggests that T_H17 cells can assume different functions depending on their cytokine profile. Therefore, T_H17 targeting is expected to have different outcomes depending on whether the downstream signature cytokine IL-17 or upstream T_H17 cell inducers, such as IL-23 or IL-1β, which determine the heterogeneous cytokine repertoire of T_H17 cells, are blocked. This has important implications for therapeutic strategies.

We have now demonstrated that T_H17 cells, which can coproduce IL-10, have regulatory functions comparable with those of Treg cells because of their ability to suppress T helper cell proliferation. In addition, they have the ability to downregulate proinflammatory innate cytokine production by monocytes, an anti-inflammatory property that Treg cells do not possess.[21] However, T_H17 cells, which were generated in the presence of IL-1β, did not have these properties. This demonstrates the existence of 2 functionally distinct types of T_H17 cell subsets, which,

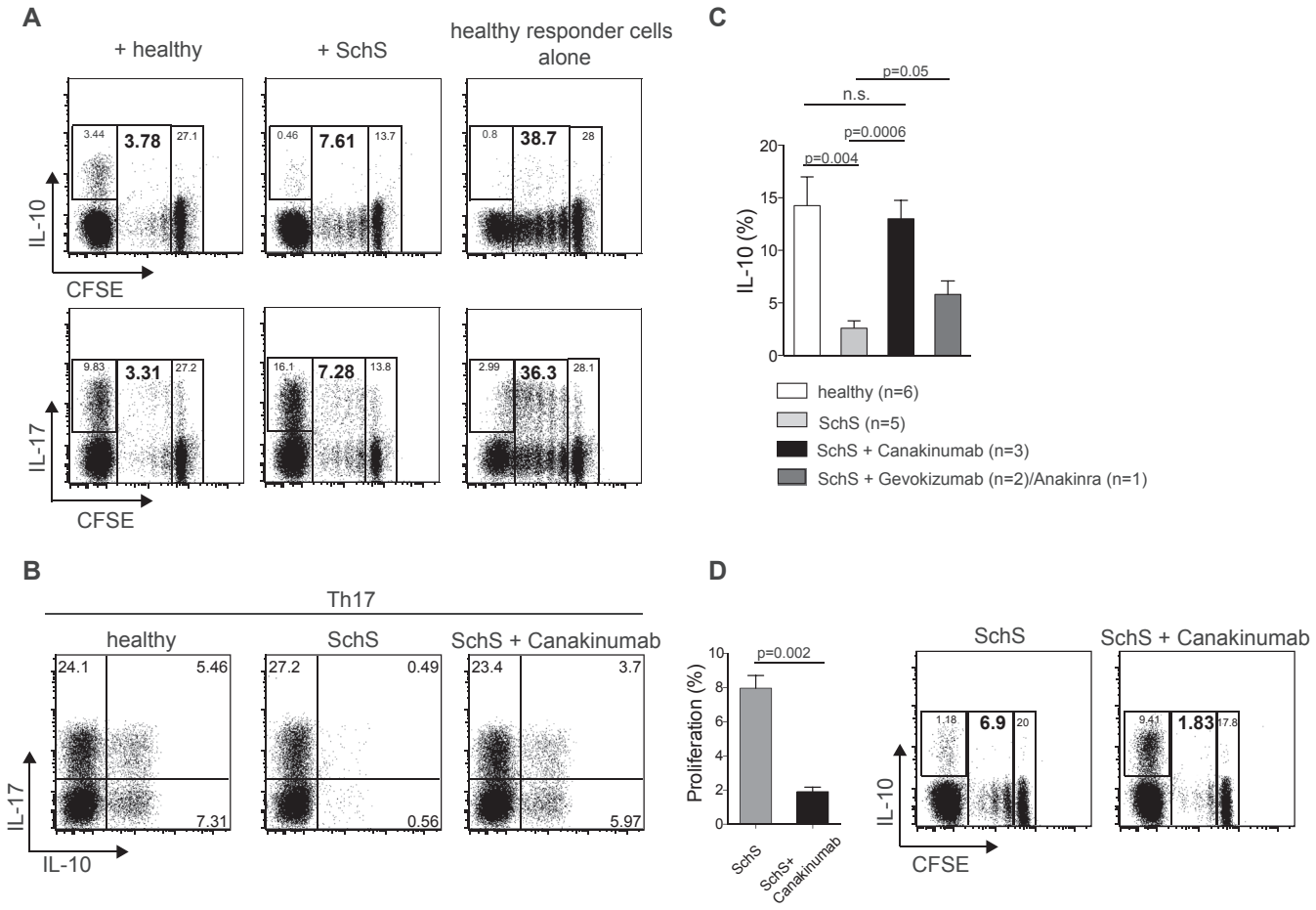

FIG 5. Regulatory properties of T_H17 cells are abrogated in patients with Schnitzler syndrome but can be restored upon IL-1β–blocking treatments. **A** and **D,** Suppression assays with T_H17 cells from healthy donors and patients with Schnitzler syndrome *(SchS)* before and after treatment with canakinumab were performed by using allogeneic, healthy, naive, CFSE-labeled responder cells. *Numbers in vertical gates* indicate percentages of IL-10^+ or IL-17^+ cells *(small regular font)* and percentages of proliferating cells *(large boldface font)* among all acquired cells. Dot plot presentation was chosen to simultaneously demonstrate proliferation by CFSE dilution, as well as cytokine expression of CFSE-unlabeled T_H17 cells, which served as suppressor cells. Data are representative of 3 experiments. **B** and **C,** Intracellular cytokine staining and flow cytometric analysis of T_H17 cells from healthy donors, symptomatic patients with Schnitzler syndrome, and the identical patients (matched pairs) after at least 6 months of treatment with canakinumab with a complete clinical response. *Numbers in gates* indicate percentages as in Fig 5, *A*. Data are representative of 3 experiments (3 matched patient pairs before and after treatment; Fig 5, *C*) or cumulative data (healthy control subjects [n = 6], patients with Schnitzler syndrome without IL-1β–blocking treatment [n = 5], and patients with Schnitzler syndrome with canakinumab treatment [n = 3], including additional treatment conditions with gevokizumab [n = 2] and anakinra [n = 1]; Fig 5, *B*). *Error bars* indicate mean ± SEMs. IL-10 expression was analyzed by using intracellular cytokine staining and FACS analysis of T_H17 cells stimulated for 48 hours with CD3/CD28 mAbs on day 5 of culture, just like patient samples from canakinumab-treated patients.

due to differential IL-10 coexpression patterns that can be modulated by IL-1β, possess either proinflammatory or anti-inflammatory functions. This provides a functional basis for the previous phenotypic characterization of IL-10–producing human T_H17 cells.[4] Therefore, the view of T_H17 cells as major culprits of autoimmune pathogenesis needs to be refined to take into consideration a novel functionality with opposite effects.

Having T_H17–IL-10^+ cells join the family of Treg helper cell subsets elicits the question of the differential contribution of T_H17 cells to immunoregulation as compared to other Treg cell subsets. IL-10 is a regulatory cytokine shared by Treg cells, T_R1 cells, and T_H17–IL-10^+ T cells. Although Treg cells are professional anti-inflammatory T cells based on several mechanisms beyond IL-10 production,[16,22] T_R1 and T_H17–IL-10^+ T cells share coexpression of IL-10 with the proinflammatory cytokines IFN-γ or IL-17, respectively.[23] Although the functional significance of simultaneous IFN-γ and IL-10 coexpression in T_R1 cells has not been clarified, T_H17–IL-10^+ cells show a kinetic segregation of IL-17 and IL-10 expression upon activation at the single-cell level.[4] This allows early proinflammatory IL-17 production upon antigen recognition, which is followed by IL-10 upregulation as a self-regulatory feedback loop.[4] This could potentially limit bystander tissue inflammation through IL-10–mediated downregulation of antigen presentation and costimulation by antigen-presenting cells and, as we have now demonstrated, proinflammatory cytokine production. Treg cells have been

reported to acquire the ability to express proinflammatory cytokines in inflammatory microenvironments. This has been interpreted as instability or plasticity, including loss of regulatory activities.[24] However, more recent studies have demonstrated that regulatory functions in Treg cells could be stably maintained despite IL-17 coexpression.[25] This distinguishes IL-10$^+$IL-17$^+$ Treg cells from IL-10$^+$IL-17$^+$ T_H17 cells on a functional level, despite similar phenotypes. In addition, we showed that the anti-inflammatory ability to suppress proinflammatory cytokine production by innate cells is a property of T_H17–IL-10$^+$ cells but not Treg cells.[21]

Overproduction of IL-1β causes considerable inflammation and tissue damage, as seen in patients with mutations in the inflammasome who suffer from autoinflammatory syndromes.[18] Therefore mechanisms must be in place to curtail inflammasome activation. While a lot of emphasis has been placed on modes and mechanisms of inflammasome activation so far, little is known on how to suppress it. Recently, T cells have been reported to dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes through cell contact–dependent mechanisms.[26] We have now demonstrated that a subset of human T_H17 cells, IL-1β–independent T_H17 cells (T_H17–IL-10$^+$), can efficiently suppress innate IL-1β secretion through their ability to produce IL-10. This shows that human T_H17 cells can also limit innate inflammation. In the future, it will be interesting to dissect the mechanistic details of how T_H17 cells affect distinct components of the inflammasome machinery to curtail IL-1β secretion.

We further showed that in the autoinflammatory disorder Schnitzler syndrome, the ability of T_H17 cells to produce IL-10 is reduced. This is consistent with overexuberant inflammasome activation and thus IL-1β secretion in autoinflammation, which cannot be sufficiently counterbalanced due to abrogated T_H17–IL-10$^+$ cell–mediated feedback mechanisms.

In conclusion, we provide novel insights into the immunoregulation of autoinflammatory syndromes by demonstrating loss of IL-10 production by T_H17 cells, which could be reversed by therapeutic IL-1β targeting. This could have implications for the pathogenesis of autoinflammatory and other chronic inflammatory diseases, as well as for future therapeutic strategies, because IL-10$^+$ and IL-10$^-$ T_H17 subsets exist with self-regulatory versus proinflammatory functions, respectively. Therefore the contribution of T_H17 cells to the pathogenesis of different diseases should be assessed considering their existence as 2 functionally distinct subsets.

We thank Dr Anna Simon, internist at the Radboud University Medical Center in Nijmegen, The Netherlands, for patient recruitment.

Key messages

- **This is the first report of functional alterations of human T_H17 cells in patients with autoinflammatory syndromes, such as Schnitzler syndrome.**
- **Systemic IL-1β blockage can restore anti-inflammatory functionalities in human T_H17 cells and thus could potentially serve as a therapeutic target in T_H17-driven inflammatory diseases, even beyond autoinflammatory syndromes.**
- **In autoinflammatory syndromes, such as Schnitzer syndrome, overproduction of IL-1β shifts the balance from anti-inflammatory to proinflammatory T_H17 cell functionalities.**

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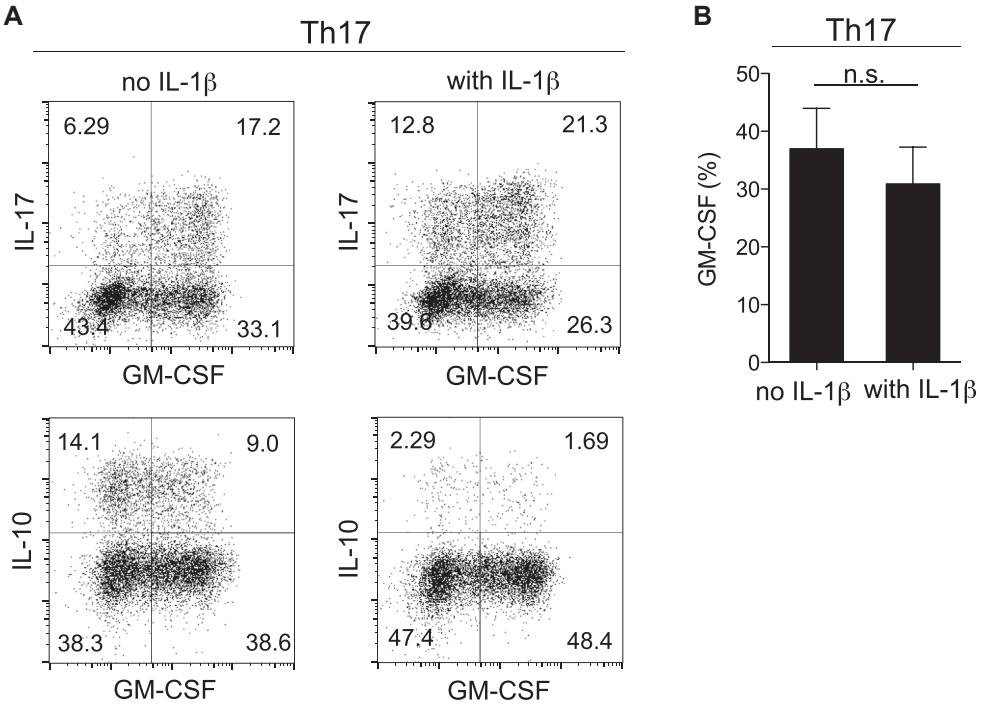

FIG E1. IL-10$^+$ and IL-10$^-$ T_H17 cells both coexpress proinflammatory GM-CSF and IL-17. **A,** T_H17 cells were isolated as CXCR3$^-$CCR4$^+$CCR6$^+$ T cells and restimulated in the presence (T_H17–IL-10$^-$) or absence (T_H17–IL-10$^+$) of IL-1β (10 ng/mL) for 48 hours with plate-bound CD3 and CD28 mAbs. On day 5, intracellular staining and flow cytometry after phorbol 12-myristate 13-acetate and ionomycin stimulation for 5 hours was performed. Shown is 1 representative experiment out of 8 experiments. **B,** Cumulative data (n = 8, mean ± S.E.M). *n.s.*, Not significant (Student *t* test).

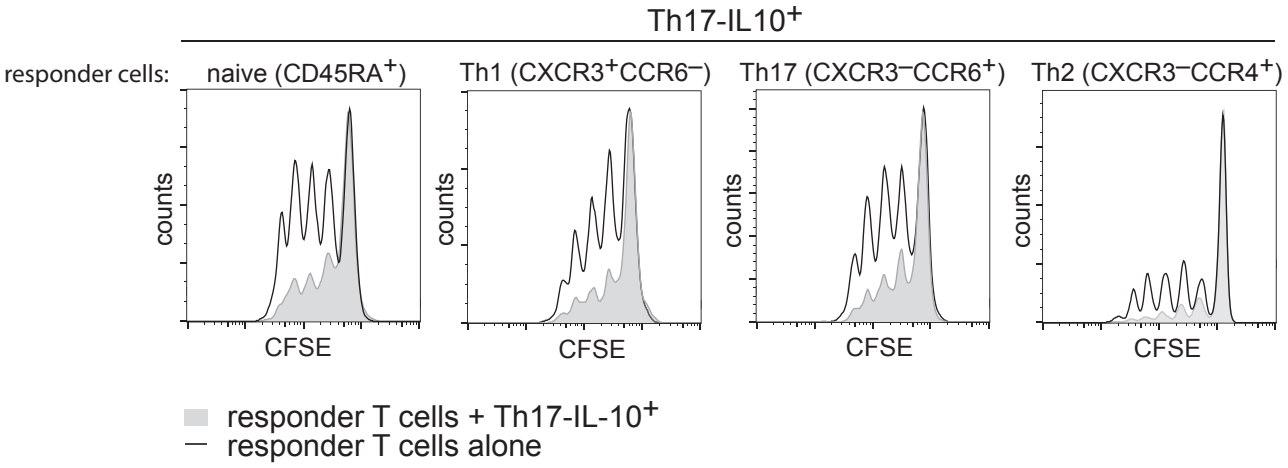

FIG E2. T_H17–IL-10^+ cells can suppress proliferation of distinct T helper cell subsets. T_H17 cells were isolated as $CXCR3^-CCR4^+CCR6^+$ T cells and restimulated for 48 hours with plate-bound CD3 and CD28 mAbs and expanded for another 3 days, which gave rise to T_H17–IL-10^+ cells as shown previously.[4] T_H17–IL-10^+ cells were then used for suppression assays with naive $CD45RA^+$ CFSE-labeled T helper cells from the same donors or responder cells isolated as distinct T helper cell subsets according to the indicated expression of chemokine receptor surface markers. CFSE dilution was assessed on day 4 by FACS. Representative data shown are from 1 donor for naive, T_H1, and T_H17 responder cells and from another donor for T_H2 responder cells because cell number limitations from buffy coats limited the simultaneous analysis of more than 3 different responder cell subsets with T_H17–IL-10^+ cells as suppressor cells. Data are representative of 5 suppression assays with naive, T_H1, and T_H17 cells as responder cells and 4 experiments for T_H2 cells as responder cells. In 2 experiments T_H2 cells are defined as $CCR4^+CXCR3^-$ cells and in the other 2 experiments additionally as $CCR6^-$ cells. In all experiments T_H17–IL-10^+ cells suppressed proliferation of their respective responder cells, although the degree of suppression varied from experiment to experiment, as did the degree of the respective control responder cell proliferation in response to OKT3 and feeder cell stimulation in the absence of T_H17–IL-10^+ suppressor cells.

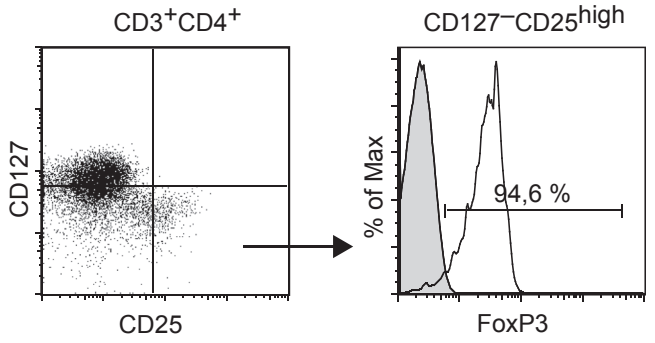

FIG E3. Phenotypic characteristics of Treg cells in suppression assays. Treg cells were isolated as $CD4^+$ T cells by means of MACS separation and FACS sorted as $CD127^-$ and $CD25^{high}$. Shown is the representative expression of forkhead box protein 3 *(FoxP3)* of this cell population, as determined by intracellular staining and flow cytometry followed by analysis with FlowJo software.

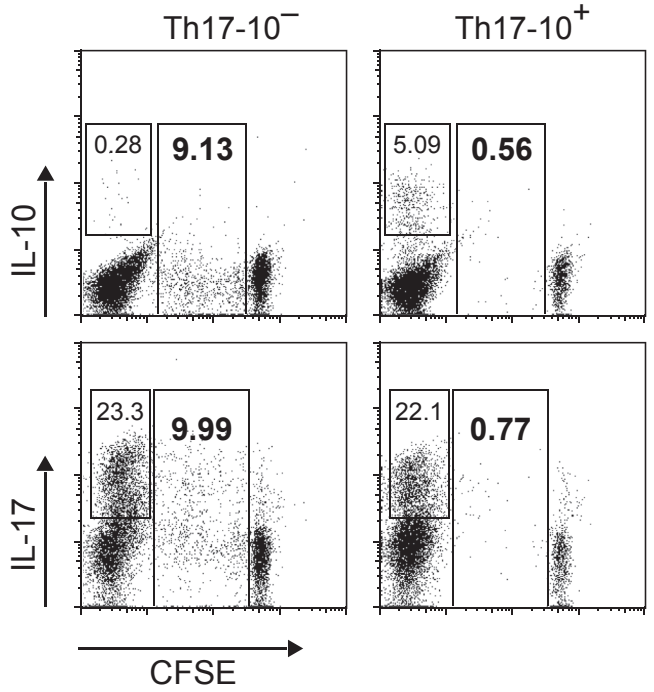

FIG E4. Differential expression of IL-10 by T_H17 cells determines their suppressive properties. T_H17 cells were isolated as $CXCR3^-CCR4^+CCR6^+$ T cells and restimulated in the presence (T_H17–IL-10^-) or absence (T_H17–IL-10^+) of IL-1β (10 ng/mL) for 48 hours with plate-bound CD3 and CD28 mAbs. T_H17–IL-10^- and T_H17–IL-10^+ cells were then used for suppression assays with naive CFSE-labeled T helper cells from the same donors. CFSE dilution was assessed on day 4 by FACS (see also Fig 3). *Numbers in quadrants* indicate percentages of positive cells, as determined by intracellular cytokine staining. Data are representative of 6 experiments.

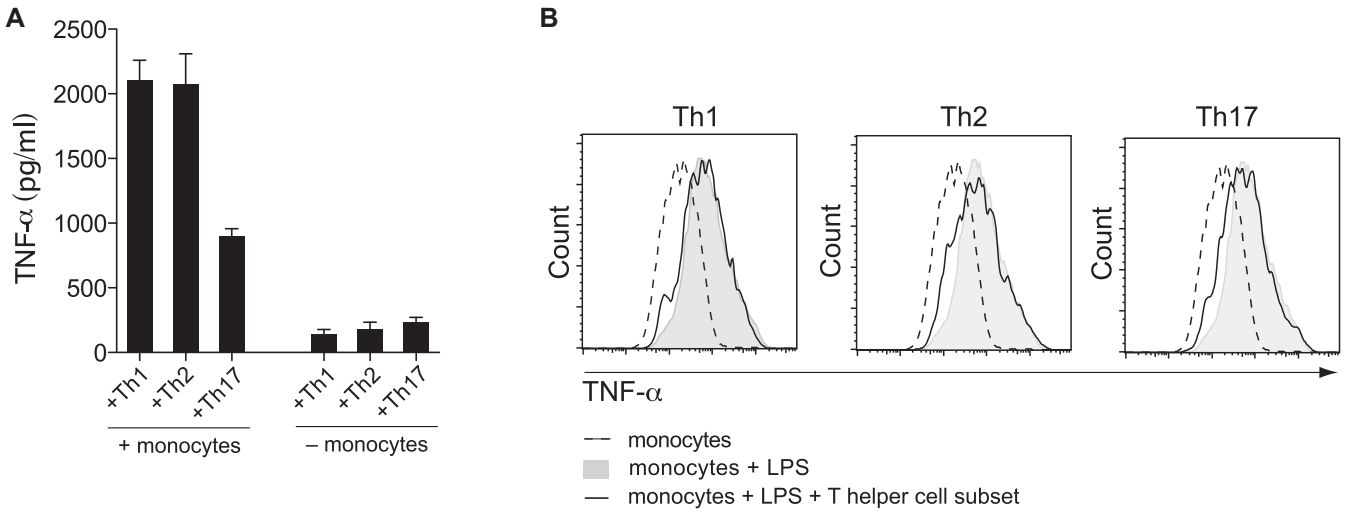

FIG E5. T_H17 cells are potent suppressors of TNF-α secretion by monocytes. **A,** Monocytes were stimulated with LPS for 48 hours and cocultured with T_H1, T_H2, and T_H17 cell subsets that have been isolated according to differential expression of CXCR3, CCR4, and CCR6 and preactivated for 48 hours with CD3 and CD28 mAbs before coculture for 48 hours. Cumulative TNF-α was analyzed by using a cytometric bead array and ELISA. The same T-cell subsets were stimulated for 48 hours with CD3 and CD28 mAbs in the absence of monocytes, washed, and analyzed for TNF-α secretion 48 hours later to determine the contribution of T cell–derived TNF-α levels in the supernatant. Data show mean ± SEMs (n = 3). **B,** Intracellular staining and flow cytometry of $CD14^+$ gated monocytes stimulated as in Fig E5, *A*, in the presence of brefeldin A during the culture period. Data are representative of 3 donors.

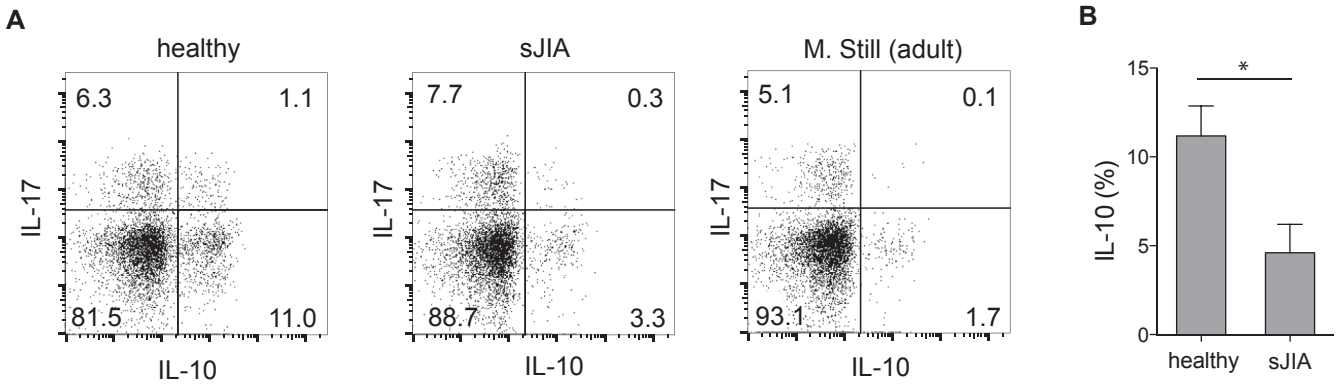

FIG E6. IL-10 expression levels in T cells from patients with Still disease (systemic juvenile idiopathic arthritis). **A,** Memory T cells were isolated by means of FACS sorting as $CD45RA^{-}CD4^{+}CCR6^{+}CXCR3$ T cells and stimulated with plate-bound CD3 and CD28 mAbs for 48 hours and expanded for another 3 days before intracellular cytokine staining after phorbol 12-myristate 13-acetate and ionomycin stimulation. Shown are representative FACS plots for 3 patients with systemic juvenile idiopathic arthritis and 1 patient with adult-onset Still disease who were not receiving IL-1β–blocking treatments. *Numbers in quadrants* indicate percentages of positive cells, as determined by intracellular cytokine staining. **B,** Cumulative data for patients with systemic juvenile idiopathic arthritis. Data show mean ± SEMs (n = 3). $*P < .03$ (Student *t* test).