Arthritis & Rheumatology
Vol. 74, No. 1, January 2022, pp 150–162
DOI 10.1002/art.41913

American College of Rheumatology
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Effect of Clonally Expanded PD-1^{high}CXCR5–CD4+ Peripheral T Helper Cells on B Cell Differentiation in the Joints of Patients With Antinuclear Antibody–Positive Juvenile Idiopathic Arthritis

Jonas Fischer,[1] Johannes Dirks,[1] Julia Klaussner,[1] Gabriele Haase,[1] Annette Holl-Wieden,[1] Christine Hofmann,[1] Stephan Hackenberg,[1] Hermann Girschick,[2] and Henner Morbach[1]

Objective. Antinuclear antibody (ANA)–positive juvenile idiopathic arthritis (JIA) is characterized by synovial B cell hyperactivity, but the precise role of CD4+ T cells in promoting local B cell activation is unknown. This study was undertaken to determine the phenotype and function of synovial CD4+ T cells that promote aberrant B cell activation in JIA.

Methods. Flow cytometry was performed to compare the phenotype and cytokine patterns of PD-1^{high}CD4+ T cells in the synovial fluid (SF) of patients with JIA and T follicular helper cells in the tonsils of control individuals. *TCRVB* next-generation sequencing was used to analyze T cell subsets for signs of clonal expansion. The functional impact of these T cell subsets on B cells was examined in cocultures in vitro.

Results. Multidimensional flow cytometry revealed the expansion of interleukin-21 (IL-21) and interferon-γ (IFNγ)–coexpressing PD-1^{high}CXCR5–HLA–DR+CD4+ T cells that accumulate in the joints of ANA-positive JIA patients. These T cells exhibited signs of clonal expansion with restricted T cell receptor clonotypes. The phenotype resembled peripheral T helper (Tph) cells with an extrafollicular chemokine receptor pattern and high T-bet and B lymphocyte–induced maturation protein 1 expression, but low B cell lymphoma 6 expression. SF Tph cells, by provision of IL-21 and IFNγ, skewed B cell differentiation toward a CD21$^{low/-}$CD11c+ phenotype in vitro. Additionally, SF Tph cell frequencies correlated with the appearance of SF CD21$^{low/-}$CD11c+CD27–IgM– double-negative (DN) B cells in situ.

Conclusion. Clonally expanded CD4+ Tph cells accumulate in the joints of ANA-positive JIA patients and, in particular, promote CD21$^{low/-}$CD11c+ DN B cell differentiation. The expansion of Tph cells and DN B cells might reflect the autoimmune response in the joints of ANA-positive JIA patients.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common childhood rheumatic disease and is characterized by synovial lymphocyte infiltration and progressive joint destruction (1). Findings from functional and genetic analyses indicate that CD4+ T helper cells may play a central role in JIA pathogenesis (2). Additionally, autoantibodies (e.g., antinuclear antibodies [ANAs]) can be detected in ~50% of patients with JIA, and the presence of ANAs is correlated with synovial lymphoid neogenesis and B cell hyperactivity (3). However, the mechanisms that promote the aberrant activation of autoreactive B cells in JIA are still poorly understood.

In JIA patients, the synovial B cell compartment mainly consists of activated memory B cells and, among these, CD21$^{low/-}$CD11c+CD27–IgM– double-negative (DN) B cells seem to be preferentially expanded in the joints of ANA-positive patients (3–7). CD21$^{low/-}$CD11c+ B cells constitute a discrete B cell population that expresses transcription factor T-bet, accumulates in inflamed tissue, and is expanded in many

Supported by the German Research Foundation (grant MO 2160/4-1). Dr. Dirks' work was supported by the Interdisciplinary Center for Clinical Research Würzburg.

[1]Jonas Fischer, Johannes Dirks, MD, Julia Klaussner, Gabriele Haase, Annette Holl-Wieden, MD, Christine Hofmann, MD, Stephan Hackenberg, MD, Henner Morbach, MD: University Hospital Würzburg, Würzburg, Germany; [2]Hermann Girschick, MD: Vivantes Klinikum im Friedrichshain, Berlin, Germany.

Mr. Fischer and Dr. Dirks contributed equally to this work.

Author disclosures are available at https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fart.41913&file=art41913-sup-0001-Disclosureform.pdf.

Address correspondence to Henner Morbach, MD, Pediatric Immunology, Department of Pediatrics, University Hospital Würzburg, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany. Email: morbach_h@ukw.de.

Submitted for publication December 18, 2020; accepted in revised form June 29, 2021.

autoimmune diseases (8,9). The subset of CD21$^{low/-}$CD11c+ B cells in humans resembles age-associated B cells in mice, which have been shown to play a central role in the pathogenesis of murine lupus (10). The human CD21$^{low/-}$CD11c+ B cell population often overlaps with CD27–IgD– DN B cells or atypical memory B cells that are expanded in settings of chronic autoantigen/antigen exposure (11,12). CD21$^{low/-}$CD11c+ DN B cell differentiation seems to depend on the interaction of these cells with CD4+ T helper cells and their secreted cytokines (7,10,12,13). However, which T helper cell subsets may promote pathogenic B cell responses at the site of inflammation in JIA patients is not yet known.

T follicular helper (Tfh) cells have been implicated in the pathogenesis of many autoimmune diseases (14,15). They are characterized by the lineage-defining transcription factor Bcl-6, high expression of programmed death 1 (PD-1), and the chemokine receptor CXCR5 that recruits them into the follicles of secondary lymphoid organs (16). These cells exert their specific "B helper" function through the secretion of cytokines (e.g., interleukin-21 [IL-21]) and by production of costimulatory molecules (e.g., inducible T cell costimulator, OX40, CD154) (17). In contrast to classic Tfh cells, which act in the germinal centers of secondary lymphoid organs, extrafollicular or peripheral T helper (Tph) cell subsets have been detected in the inflamed joint tissue of patients with autoimmune diseases, and also have been observed in various murine models of autoimmune disease (18,19). In particular, Tph cells that accumulated in the joints of patients with seropositive rheumatoid arthritis (RA) also secreted IL-21 but did not express Bcl-6 and CXCR5, and therefore Tph cells in RA seem to differ from classic Tfh cells (20). Interestingly, IL-21–expressing CD4+ T helper cells could also be detected in the inflamed joints of ANA-positive JIA patients; however, neither the phenotype nor the function of this T helper cell subset has been investigated in detail to date (21). Therefore, in this study, we investigated the occurrence and phenotype of CD4+ T cells that exhibit a "B helper" function at the site of inflammation in JIA patients and examined the functional impact of these cells on B cell differentiation.

PATIENTS AND METHODS

Patients. This study included 53 patients with active JIA, in whom joint puncture had been performed for intraarticular steroid injection (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). All patients were followed up at the Children's Hospital at the University Hospital of Würzburg. Human palatine tonsil samples were obtained from patients undergoing tonsillectomy due to recurrent tonsillitis and/or tonsillotomy due to tonsillar hypertrophy. Written informed consent was obtained from the patients' legal guardians. The study protocol was reviewed by the Research Ethics Committee of the University of Würzburg (approval no. 299/17) and was conducted in accordance with the Declaration of Helsinki.

Sample preparation. Synovial fluid (SF) and peripheral blood (PB) samples were collected in EDTA tubes. Mononuclear cells were isolated using Ficoll density-gradient centrifugation. Tonsil mononuclear cells were obtained using mechanical disaggregation followed by Ficoll density-gradient centrifugation. Cells were stored in liquid nitrogen until used.

Flow cytometry and cell sorting. Details on the flow cytometry and cell sorting procedures and antibodies used are included in Supplementary Materials and Methods (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

***TCRVB* repertoire sequencing.** SF CD4+CD45RO+CXCR5–PD-1^{high}HLA–DR+ and CD4+CD45RO+CXCR5–PD-1$^{low/-}$HLA–DR– T cells obtained from 4 ANA-positive JIA patients were sorted using flow cytometry. Gene sequencing was performed on each sorted cell subset to identify the *TCRVB* repertoire. Additional details are included in Supplementary Materials and Methods (available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). This targeted locus study project has been deposited at DataBank of Japan/ European Nucleotide ArchiveA/GenBank under the accession no. KEXF00000000. The version described in this report is the first version, accession no. KEXF01000000.

T cell/B cell coculture. A total of 30,000 sorted SF or tonsil CD4+PD-1+ T cells were cocultured with sorted healthy control PB CD19+CD27+IgM– B cells at a 1:1 ratio and were stimulated with staphylococcal enterotoxin B (0.1 μg/ml) in a 96-well plate in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. For blocking experiments, 10 μg/ml of IL-21 receptor–Fc (R&D Systems), 10 μg/ml of anti–interferon-γ (anti-IFNγ) (cloneB27; BioLegend), and/or 0.1 μg/ml of ruxolitinib (InvivoGen) was added to the cultures. After 7 days, B cell differentiation was analyzed using flow cytometry, and total IgG concentrations in culture supernatants were measured using an in-house enzyme-linked immunosorbent assay (22). To analyze T-bet induction in B cells, T cells were sorted as described above and were cocultured for 48 hours with allogenic B cells that had been immunomagnetically purified from the PB of healthy controls using CD20 microbeads (Miltenyi Biotec). B cells were additionally stimulated with F(ab')$_2$–anti-IgM (5 μg/ml; The Jackson Laboratory), and in CD19+CD27– gated naive B cells, intracellular T-bet expression was analyzed using flow cytometry.

B cell activation assays. B cells were purified from the PB of healthy controls using CD20 microbeads (Miltenyi Biotec). A total of 30,000 purified B cells were cultured in a 96-well plate

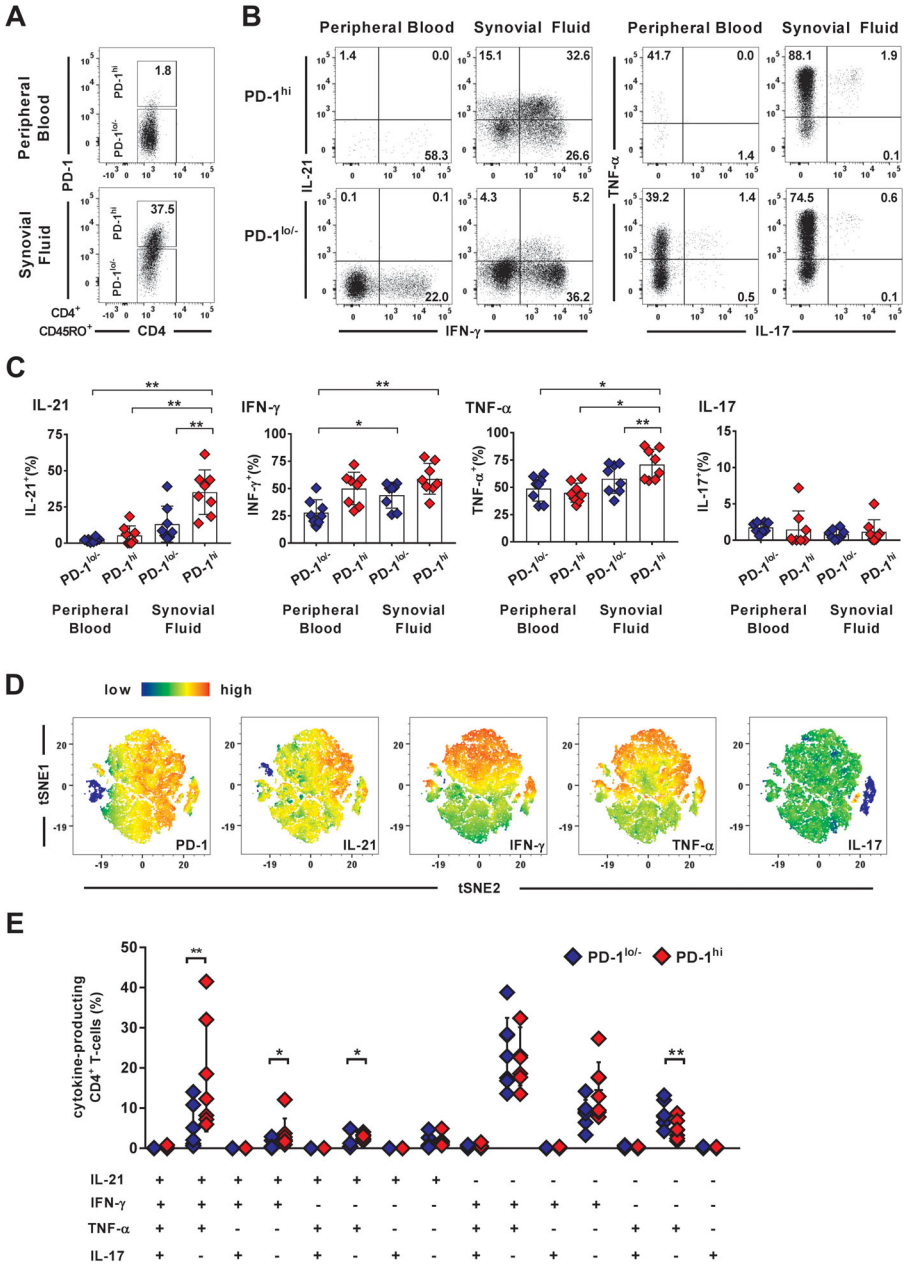

Figure 1. CD4+ T cells with high expression of programmed death 1 (PD-1^{high}) are characterized by coexpression of interleukin-21 (IL-21) and interferon-γ (IFNγ) in the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA). **A**, Representative dot plots showing PD-1 expression on matched peripheral blood (PB) and SF CD45RO+CD4+ T cells from 1 patient with JIA. **B** and **C**, Representative dot plots (**B**) and the mean frequencies of cytokine-expressing cells (**C**) within CD45RO+PD-1$^{low/-}$ or CD45RO+PD-1^{high} CD4+ T cells from the PB or SF of JIA patients. **D**, Heatmaps of merged data derived from t-distributed stochastic neighbor embedding (t-SNE) analysis showing expression of PD-1, IL-21, IFNγ, tumor necrosis factor (TNF), and IL-17 on CD4+ T cells from the SF of 5 JIA patients. Colors indicate the expression level of each marker. **E**, Polyfunctional cytokine expression data in SF PD-1^{high} or PD-1$^{low/-}$ CD4+ T cells. Percentages of CD4+ T cells displaying each cytokine coexpression pattern in the SF of 7 JIA patients were determined using flow cytometry, with a Boolean gating strategy. In **C** and **E**, symbols represent individual samples; bars in **C** show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$, by one-way analysis of variance with Tukey's multiple comparisons test for comparisons between >2 groups (**C**) and paired *t*-test for comparisons between 2 groups (**E**). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract.

in 200 μl of RPMI 1640 medium supplemented with 10% FCS and penicillin/streptomycin in the presence of CD40L (5 μg/ml) (BioLegend), with the addition of either IFNγ or IL-21 or both (each at a concentration of 100 ng/ml). After 2 days, intracellular expression of T-bet in CD19+CD27− gated naive B cells was analyzed using flow cytometry.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software version 8.0. Hierarchical cluster analysis and principal components analysis were performed using ClustVis. Data are expressed as scattered individual values and the mean ± SD. Either Student's 2-tailed t-test or one-way analysis of variance with Tukey's multiple comparisons test was used

to compare data sets with either 2 or >2 continuous variables, respectively. Pearson's correlation coefficient was used to analyze the correlation between variables. A chi-square test with Yates' correction was used for contingency tables. *P* values less than 0.05 were considered significant.

RESULTS

Correlation of high expression of PD-1 on SF CD4+ T cells with a distinct cytokine pattern characterized by coexpression of IL-21 and IFNγ. We used PD-1 as a surrogate surface marker to explore the SF CD4+ T cell compartment in the joints of patients with JIA for the presence of activated T helper cells with a potential B helper function (e.g., IL-21 expression). The SF CD45RO+CD4+ memory T cell compartment was significantly enriched in PD-1^{high} cells as compared to the CD45RO+CD4+ memory T cell compartment in matched PB samples from each individual patient (mean ± SD 3.0 ± 1.7% in the PB versus 34.4 ± 19.0% in the SF; $P < 0.001$) (Figure 1A). We then investigated whether these PD-1^{high}CD4+ T cells express a distinct cytokine pattern characteristic of B helper cells or whether the high expression of PD-1 in these cells could be indicative of a state of exhaustion (23).

We therefore assessed the capacity of SF PD-1^{high}CD4+ T cells to express IL-21, IFNγ, tumor necrosis factor (TNF), and IL-17 upon restimulation (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). In the SF from patients with JIA, the PD-1^{high} T cell subset was particularly enriched in IL-21–expressing cells, whereas there was only a slight difference in the cytokine patterns seen between SF and PB PD-1$^{low/–}$ T cells and PB PD-1^{high} T cells. Moreover, compared to these other cell subsets, the SF PD-1^{high} T cell population contained high levels of IFNγ-expressing and significantly higher levels of TNF-expressing cells, but similar levels of IL-17–expressing cells (Figures 1B and C). Indeed, up to 40% of SF PD-1^{high} cells showed high expression of IL-21, and >50% of this cell subset showed increased expression of IFNγ and TNF, suggesting that within this cell population, there may be a potential overlap between the "B helper" cytokine signature (i.e., increased levels of IL-21) and the Th1 cytokine signature (i.e., increased levels of IFNγ and TNF) (Figures 1B and C).

To strengthen this hypothesis, we performed an unsupervised analysis of multiparameter flow cytometry–based cytokine expression data derived from the SF CD4+ T cells obtained from 5 JIA patients. A t-distributed stochastic neighbor embedding analysis of SF CD4+ T cells revealed an island of IL-21–expressing T cells that was characterized by high expression of PD-1 together with high expression of IFNγ and TNF, but not IL-17 (Figure 1D). Using a Boolean gating strategy, we further analyzed polyfunctional cytokine combinations at the single-cell level within PD-1^{high} and PD-1$^{low/–}$ SF CD4+ T cells obtained from 7 additional JIA patients. CD4+ T cells that expressed IL-21 together with IFNγ and/or TNF, but not IL-17, were more enriched in PD-1^{high} cells compared to PD-1$^{low/–}$ cells (Figure 1E). Of note, the cytokine pattern of SF PD-1^{high}CD4+ T cells was divergent from classic Tfh cells, since expression of IFNγ and/or TNF could not be observed within IL-21–expressing PD-1^{high}CD4+ T cells derived from tonsil tissue, which, in contrast, contained higher levels of IL-4–expressing T cells and IL-10–expressing T cells compared to that observed in the SF (Supplementary Figures 2A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

Therefore, we conclude that IL-21 is a signature cytokine of SF PD-1^{high}CD4+ T cells in JIA patients, but unlike classic Tfh cells, coexpression of IFNγ and TNF also indicates skewing toward a Th1 phenotype. Furthermore, the robust capacity of SF PD-1^{high}CD4+ T cells to express cytokines upon stimulation is evidence against the presence of an exhaustive state that might be indicated by high expression of PD-1.

Signs of activation and an extrafollicular/peripheral migration pattern in PD-1^{high}CD4+ T cells accumulating at the site of inflammation. Based on the cytokine pattern, we postulated that SF PD-1^{high}CD4+ T cells differ from classic Tfh cells and PD-1$^{low/–}$ memory T helper cells and may rather constitute a distinct T effector subset. Therefore, we further aimed to delineate this T cell subset in more detail by comparing its extended phenotype to that of PD-1$^{low/–}$ memory CD4+ T cells in SF and that of classic Tfh cells in the tonsils from control individuals (Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). Indeed, principal components analysis and hierarchical clustering of a flow cytometry data set comprising 17 different markers clearly distinguished SF PD-1^{high} and PD-1$^{low/–}$ CD4+ T cells, as well as tonsil PD-1^{high}CD4+ T cells, the latter representing classic Tfh cells (Figures 2A and B). In contrast to classic Tfh cells derived from tonsils, SF PD-1^{high}CD4+ T cells lacked the chemokine receptor CXCR5 that classically characterizes Tfh cells (Figures 2B–D).

Aside from a lack of CXCR5 expression, both SF CD4+ T cell populations (PD-1^{high} as well as PD-1$^{low/–}$) significantly differed from classic Tfh cells, as evidenced by a peripheral/inflammatory and extrafollicular migration pattern with increased expression of CCR2, CCR5, and P-selectin glycoprotein ligand 1 (Figures 2B–D and Supplementary Figures 4A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). Additionally, SF PD-1^{high}CD4+ cells differed from SF PD-1$^{low/–}$ CD4+ cells and also from tonsil Tfh cells by increased expression of activation markers (e.g., HLA–DR, OX40), as well as by high expression of the Th1 transcription factor T-bet, higher expression of B lymphocyte–induced maturation protein 1 (BLIMP-1), and lower expression of Tfh lineage-defining

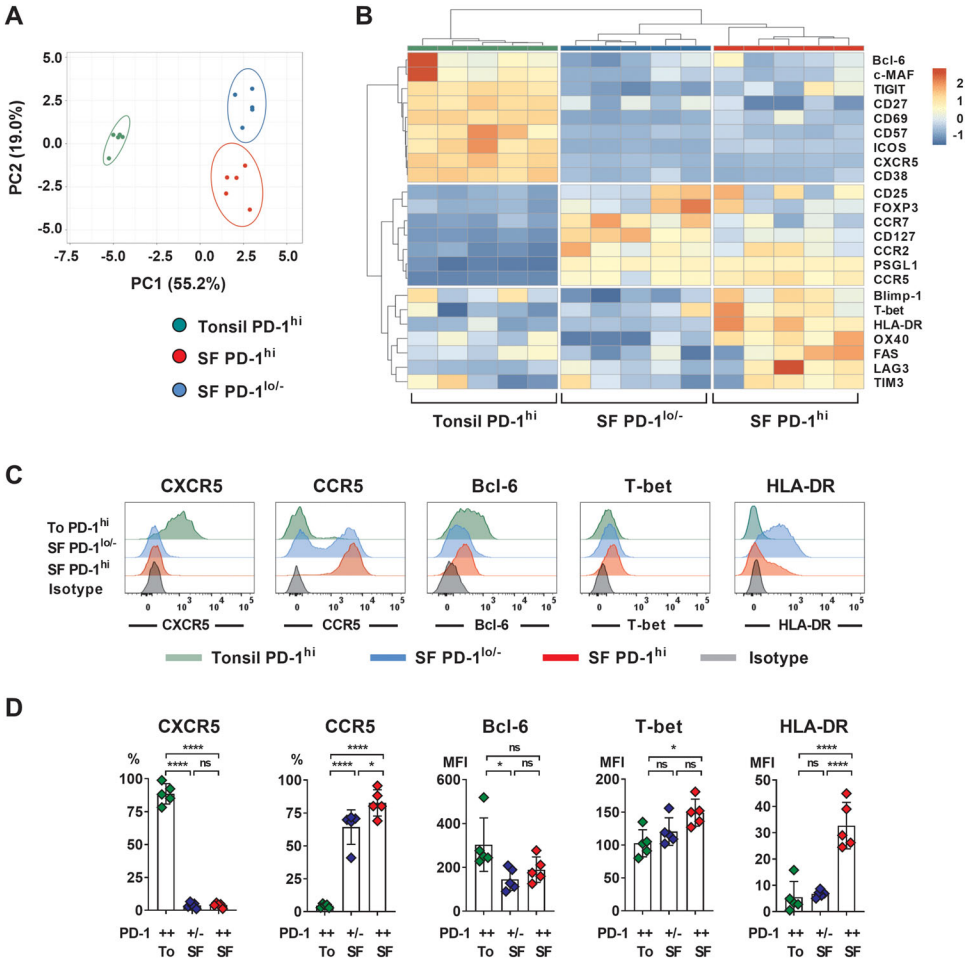

Figure 2. SF PD-1^{high} CD4+ T cells show signs of activation and express an extrafollicular/inflammatory migration pattern. **A**, Principal components (PC) analyses of flow cytometry data assessing the phenotypes of SF PD-1^{high} and PD-1$^{low/-}$ CD4+ T cells from 5 JIA patients and tonsil PD-1^{high} CD4+ T cells from 5 controls. **B**, Unsupervised hierarchical clustering analysis of the 17 different markers from the flow cytometry data set. Patterns of expression are shown as heatmaps, in which colors in each row represent the normalized marker expression, and data are split into 3 clusters. **C**, Representative histograms showing CXCR5, CCR5, Bcl-6, T-bet, and HLA–DR expression on tonsil (To) PD-1^{high} CD4+ T cells, SF PD-1$^{low/-}$ CD4+ T cells, and PD-1^{high} CD4+ T cells. **D**, Flow cytometry analysis of PD-1^{high} and PD-1$^{low/-}$ CD4+ T cells from the SF of 5 JIA patients and PD-1^{high} CD4+ T cells from the tonsils of 5 controls showing mean frequencies of marker-expressing cells or mean fluorescence intensity (MFI) of marker expression. Symbols represent individual samples; bars show the mean ± SD. * = $P < 0.05$; **** = $P < 0.0001$, by one-way analysis of variance with Tukey's multiple comparisons test. TIGIT = T cell immunoreceptor with Ig and ITIM domains; ICOS = inducible costimulator; PSGL-1 = P-selectin glycoprotein ligand 1; BLIMP-1 = B lymphocyte–induced maturation protein 1; TIM-3 = T cell immunoglobulin and mucin domain–containing protein 3; NS = not significant (see Figure 1 for other definitions).

transcription factors Bcl-6 and c-MAF (Figures 2B–D and Supplementary Figures 4A and B, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

Taken together, these markers established a signature that clearly distinguishes SF PD-1^{high}CD4+ T cells from classic Tfh cells as well as PD-1$^{low/-}$ memory T cells. Hence, the extended phenotype of SF PD-1^{high}CD4+ T cells is characterized by signs of sustained activation (i.e., enrichment of HLA–DR+ cells), an inflammatory/peripheral chemokine receptor pattern (i.e., enrichment of CXCR5– cells), and a mixed pattern of transcription factors that partially resembles that seen in Tfh cells or Th1 cells.

Since a significant proportion of pathogenic Th1 cells in the joints of JIA patients is derived from a shift of Th17 cells to nonclassic Th1 cells, which can be defined by persistent CD161 expression, we also assessed CD161 expression in SF PD-1^{high}CD4+ T cells (24–26). In contrast to tonsil PD-1^{high}CD4+ T cells that were shown to have minimal CD161 expression, a considerable percentage of SF PD-1^{high}CD4+ T cells expressed

CD161 (Figures 3A and B). We further examined IL-17–expressing and/or IL-21–expressing CD4+ T cell subsets to investigate coexpression of CD161 (Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

As expected, most of the IL-17–expressing cells were CD161+ cells, which was independent of IL-21 coexpression. However, >50% of the IL-21–expressing cells that did not coexpress IL-17 (IL-17–IL-21+) also expressed CD161 (Figures 3C and D). The majority of these IL-21+ cells (including the very rare population of IL-17+IL-21+ cells) were from the PD-1^{high} compartment, whereas the IL-17+ cells that did not coexpress IL-21 were from the PD-1$^{low/–}$ compartment (Figures 3C and D). Additionally, expression levels of the Th17-defining transcription factor retinoic acid receptor–related orphan nuclear receptor γt (RORγt) within the 3 cell subsets was similar to those of CD161, with lower but residual expression of RORγt in IL-17–IL-21+ cells (Figures 3C and D). Expression levels of RORγt, as well as CD161, within the cytokine-expressing T cell subsets were independent of IFNγ coexpression (Supplementary Figure 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). Hence, a considerable percentage of PD-1^{high}IL-21+IL-17–CD4+ T cells in the joints of JIA patients coexpress CD161, suggesting that these cells may have originated from the Th17 subset of T helper cells.

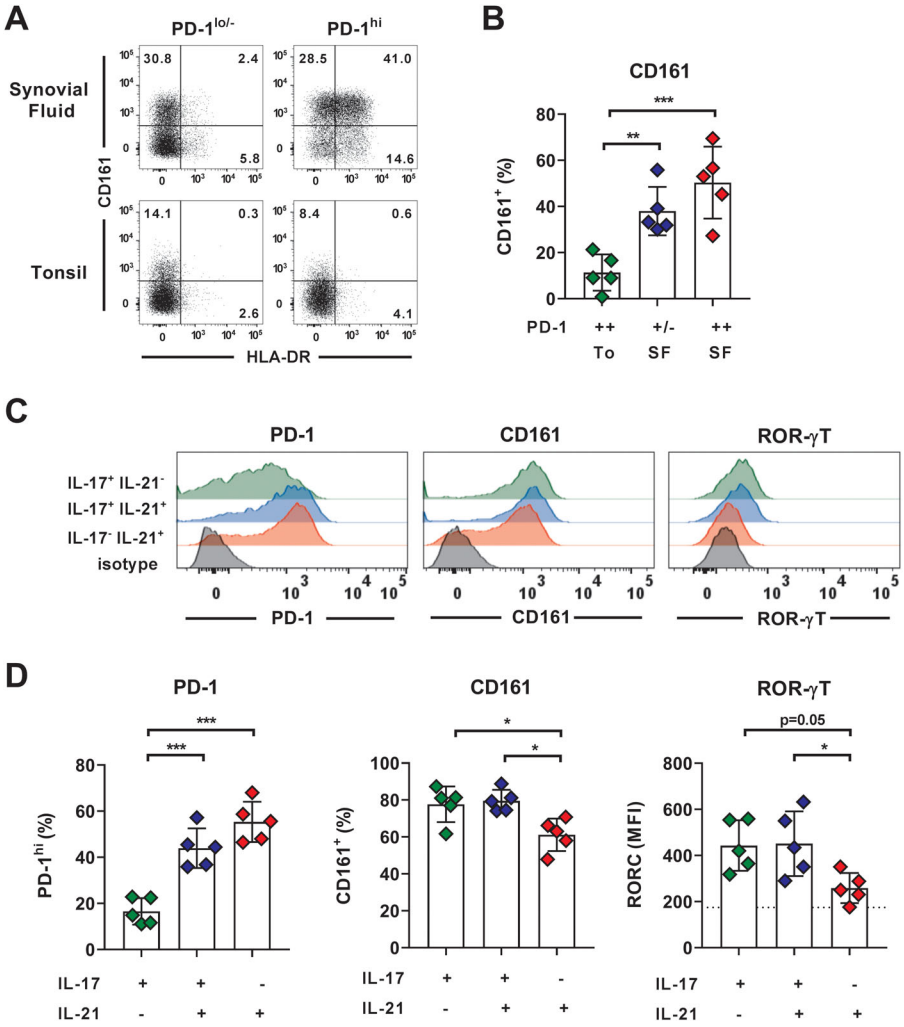

Figure 3. Coexpression of CD161 in PD-1^{high}HLA–DR+ and IL-21+CD4+ T cells from the SF of JIA patients. **A**, Representative dot plots showing CD161 and HLA–DR expression on SF and tonsil PD-1$^{low/–}$ and PD-1^{high} CD4+ T cells. **B**, Mean frequencies of CD161+ cells among PD-1^{high} and PD-1$^{low/–}$ CD4+ T cells from the SF of 5 JIA patients and PD-1^{high} CD4+ T cells from the tonsils (To) of 5 controls. **C**, Representative histograms showing PD-1, CD161, and receptor–related orphan nuclear receptor γt (RORγt) expression in SF IL-17+IL-21–, IL-17+IL21+, and IL-17–IL-21+ CD4+ T cells. **D**, Flow cytometry analysis of IL-17– and/or IL-21–expressing CD4+ T cells from the SF of 5 JIA patients, showing mean frequencies of PD-1+ or CD161+ cells or mean fluorescence intensity (MFI) of RORγt expression. In **B** and **D**, symbols represent individual samples; bars show the mean ± SD. The broken line shows the MFI of the isotype control. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, by one-way analysis of variance with Tukey's multiple comparisons test. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract.

PD-1^{high}CXCR5–HLA–DR+CD4+ T cells identified as a clonally expanded population in the SF of patients with JIA. The preferential occurrence of a subset of highly activated effector CD4+ T cells in the joints of JIA patients raises the question of whether this accumulation might be elicited by antigen-driven clonal expansion or rather a random influx into the inflamed joints. The SF PD-1^{high}CXCR5–HLA–DR+CD4+ T cell population (PD-1^{high}) was more enriched in cells expressing Ki-67 compared to the matched PD-1$^{low/-}$–HLA–DR–CD45RO+ memory cell population (PD-1$^{low/-}$) (Figure 4A), suggestive of recent proliferation in vivo and a potential antigen encounter.

Next, we performed *TCRVB* next-generation sequencing on matched SF PD-1^{high} and PD-1$^{low/-}$ T cells from 4 JIA patients (Supplementary Figure 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). Additionally, we analyzed the *TCRVB* repertoire of both T cell populations derived from 2 separate joints from 1 of these patients. Despite the fact that the number of cells in each T cell subset was equal in the matched pairs of SF samples from individual patients, the number of unique clones was lower in the PD-1^{high} T cell subset than in the PD-1$^{low/-}$ T cell subset (Supplementary Table 2, available on the *Arthritis & Rheumatology*

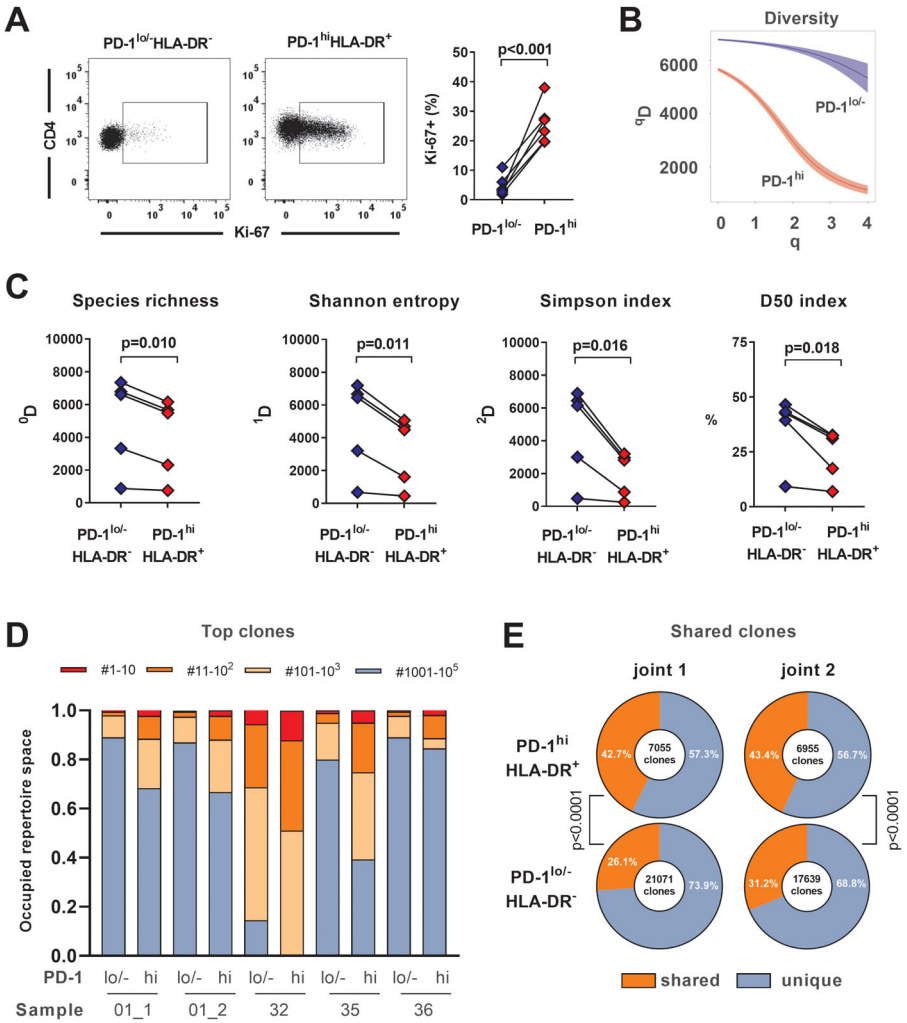

Figure 4. Signs of clonal expansion in SF PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells from patients with JIA. **A**, Representative dot plots showing Ki-67 expression and the Ki-67+ cell frequency within PD-1^{high}CXCR5–HLA–DR+ and PD-1$^{low/-}$CXCR5–HLA–DR– CD4+ T cells from the SF of 5 JIA patients. **B**, Clonal diversity analysis of the *TCRVB* repertoire in both T cell populations in 5 different joints from 4 JIA patients, using the Hill generalized diversity index. The diversity index (^{q}D) was calculated over a range of diversity orders (q) and was plotted as a smooth curve. Representative curves from 1 patient are shown. **C**, Representations of species richness (q = 0), Shannon diversity index (q = 1), Simpson diversity index (q = 2), and diversity 50 (D50) index in both T cell subsets. Symbols represent individual values of each T cell population from a single joint sample from the same patient. Significance was determined by paired t-test. **D**, Stacked bar graphs showing the clonal proportion of the top n clonotypes within each T cell subset from the joint samples from 4 JIA patients. **E**, Pie charts showing the proportions of shared clonotypes within both T cell subsets between 2 different joints from the same JIA patient. The proportion of shared clones in the PD-1^{high}CXCR5–HLA–DR+ cells was compared to that of the matched PD-1$^{low/-}$CXCR5–HLA–DR– T cell subsets from the same joint. *P* values were determined by chi-square test with Yates' correction. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract.

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We therefore analyzed the clonal diversity of each T cell subset using the Hill diversity index as a surrogate marker of clonal expansion, in which q values of 0, 1, or 2 are used, equivalent to measures of diversity such as the species richness, Shannon entropy index, and the inverse Simpson index. Clonal diversity was significantly lower in the PD-1^{high} T cell subset compared to the matched PD-1$^{low/–}$ T cell subset (Figures 4B and C). The D50 index, indicating the frequency of most abundant clones (top clones) of all clones accounting for 50% of all unique sequences, was significantly lower in the PD-1^{high} subset, and the top clones occupied more repertoire space in the PD-1^{high} subset compared to the matched PD-1$^{low/–}$ subset from the same joint sample (Figures 4C and D). These findings indicate significantly reduced clonal diversity in the PD-1^{high} cell subset, suggestive of a clonally expanded population.

When we assessed the extent of shared clonotypes of cell subsets from 2 different joints in the same patient, we observed that the PD-1^{high} cell subset showed significantly higher clonal overlap than the PD-1$^{low/–}$ subset (Figure 4E). Furthermore, the clonal overlap within samples obtained from different JIA patients was higher in the PD-1^{high} subset than in the PD-1$^{low/–}$ subset (Supplementary Figure 8A, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). Moreover, a particular set of *TCRVB* and *TCRJB* gene segments was overrepresented in the PD-1^{high} subset in each of the analyzed joint samples obtained from the JIA patients (Supplementary Figures 8B and C). Hence, the PD-1^{high} subset appears to represent a distinct population within SF CD4+

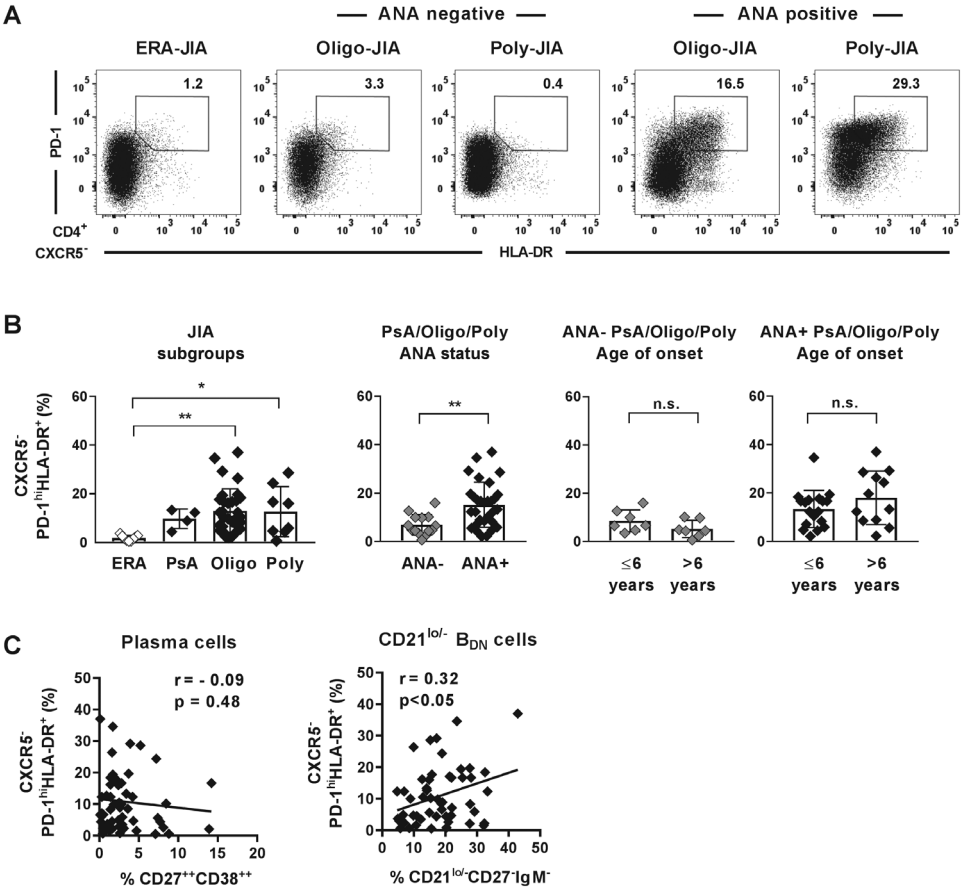

Figure 5. Accumulation of PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells in the joints of antinuclear antibody (ANA)–positive JIA patients is correlated with the proportion of CD21$^{low/–}$ double-negative (DN) B cells. **A** and **B**, Representative dot plots showing PD-1 and HLA–DR expression on SF CXCR5–CD4+ T cells from JIA patients (**A**) and the distribution of SF PD-1^{high}CXCR5–HLA–DR+ CD4+ T cell frequencies stratified according to the subgroup of JIA patients (9 patients with enthesitis-related arthritis [ERA], 32 with oligoarticular JIA [oligo-JIA], 8 with polyarticular JIA [poly-JIA], and 4 with psoriatic arthritis [PsA]–JIA), and within these subgroups (including all JIA patients except for those with ERA), stratified according to ANA status (30 ANA+ patients versus 14 ANA– patients) and age at disease onset (**B**). Symbols represent individual patients; bars show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$, by unpaired *t*-test for comparison between 2 groups and one-way analysis of variance with Tukey's multiple comparisons test for comparisons between >2 groups. **C**, Correlation between the frequency of SF PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells and frequency of SF CD27++CD38++ plasmablasts/plasma cells and CD21$^{low/–}$ CD27–IgM– DN B cells in 43 JIA patients. Correlations were determined using Pearson's correlation coefficient. See Figure 1 for other definitions.

T helper cells that is clonally expanded and potentially driven by antigens that are present at the site of inflammation.

Correlation of PD-1^{high}CXCR5–HLA–DR+CD4+ T cells in the joints of ANA-positive JIA patients with expansion of CD21$^{low/-}$CD11c+ DN B cells. The oligoclonal expansion of PD-1^{high}CXCR5–HLA–DR+CD4+ T cells secreting the B helper cytokine IL-21 at the site of inflammation raises the question of whether this T cell subset might preferentially expand in a distinct subgroup of JIA patients who display a clinical phenotype characterized by signs of B cell dysregulation, such as the presence of ANAs. Therefore, we analyzed the distribution of PD-1^{high} T cells within the SF CD4+ T cell compartment in a larger cohort of JIA patients (Supplementary Figure 9 and Supplementary Table 1,

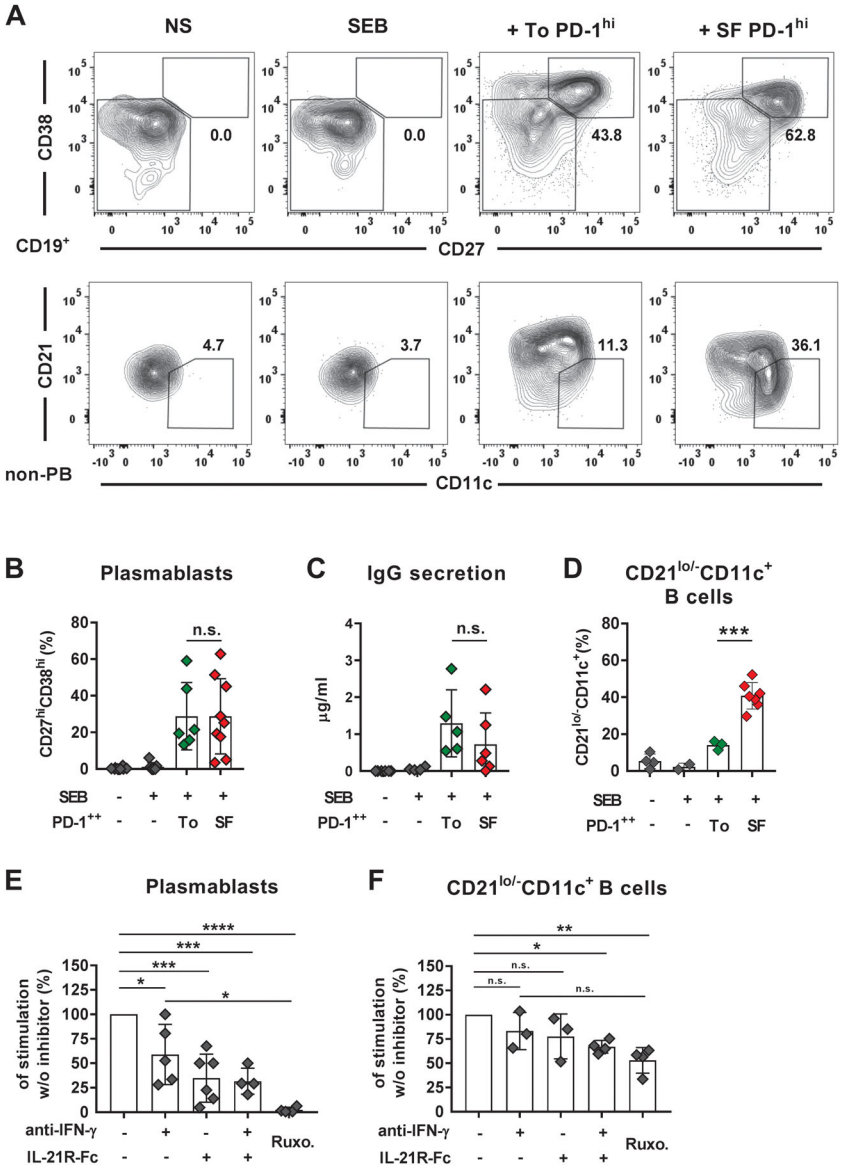

Figure 6. SF PD-1^{high} CD4+ T cells skew B cell differentiation toward a CD21$^{low/-}$CD11c+T-bet+ phenotype in vitro. **A**, Representative dot plots showing the frequencies of CD38++CD27++ plasmablasts (top) and CD21$^{low/-}$CD11c+ B cells (bottom) in T cell/B cell cocultures in vitro. Cocultures with T cells included CD19+CD27+IgM– switched memory B cells from healthy controls under conditions of no stimulation (NS), using stimulation with staphylococcal enterotoxin B (SEB) alone, or using stimulation with SEB and culturing with sorted PD-1^{high} CD4+ T cells from control tonsils (To) or SF from JIA patients. **B**–**D**, Frequencies of CD27++CD38++ plasmablasts and CD21$^{low/-}$CD11c+ B cells as well as IgG concentration in T cell/B cell cocultures. **E** and **F**, Frequencies of stimulated plasmablasts (**E**) and CD21$^{low/-}$CD11c+ B cells (**F**) in cocultures of SF PD-1^{high}CD4+ T cells from JIA patients with switched memory B cells from healthy controls in the absence or presence of IL-21 receptor–Fc, anti-IFNγ, and/or the inhibitor ruxolitinib (Ruxo.). Frequencies were determined relative to the values in stimulated cell cultures without any inhibitors (set at 100%). In **B**–**F**, symbols represent individual values; bars show the mean ± SD. * = $P < 0.05$; ** $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by unpaired Student's *t*-test for comparisons between 2 groups (**B**–**D**) and one-way analysis of variance with Tukey's multiple comparisons test for comparisons between >2 groups (**E** and **F**). Non-PB = non-plasmablast; n.s. = not significant (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract.

available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

The frequency of PD-1^{high} T cells was highest in the SF from patients with oligoarticular JIA and those with polyarticular JIA and was lowest in the SF from patients with enthesitis-related arthritis (Figures 5A and B). Since ANA-positive patients within the oligoarticular JIA, polyarticular JIA, and psoriatic arthritis–JIA group seem to constitute a clinically homogeneous group of patients characterized by an early age at disease onset (27,28), we further compared the ANA-positive patients to the ANA-negative patients. Indeed, the frequency of PD-1^{high} T cells was significantly higher within the SF of ANA-positive patients compared to ANA-negative JIA patients (Figure 5B). However, the frequencies of PD-1^{high} T cells did not correlate with age at onset ($r = 0.09$, $P = 0.57$) (data not shown), and no differences were observed between patients with early or later disease onset (Figure 5B).

Next, we used an existing flow cytometry B cell data set derived from the patients analyzed above to address the distribution of different SF B cell populations in order to explore the association between the expansion of SF PD-1^{high} T cells and distinct effector B cell subsets (5). Whereas the frequency of CD21$^{low/-}$CD11c+ DN B cells significantly correlated with that of SF PD-1^{high} T cells, the frequency of CD27++CD38++ plasmablasts/plasma cells did not show a significant correlation with PD-1^{high} T cells (Figure 5C). Therefore, we conclude that the accumulation of PD-1^{high}CXCR5–HLA–DR+CD4+ T cells together with CD21$^{low/-}$CD11c+ DN B cells represents a characteristic cellular pattern within the inflamed joints of JIA patients who are ANA positive.

B cell differentiation skewed toward CD21$^{low/-}$CD11c+ cells in vitro in the presence of SF PD-1^{high}CD4+ T cells. The correlation between SF PD-1^{high}CXCR5–HLA–DR+CD4+ T cells and CD21$^{low/-}$CD11c+ DN B cells in situ, as well as the distinct cytokine patterns of these T cells with coexpression of IL-21 and IFNγ, suggests a different functional impact on B cell differentiation than that expected from classic Tfh cells. Therefore, we utilized activation assays in vitro to compare the functional impact on B cell differentiation between this PD-1^{high}CD4+ T helper cell subset derived from SF of JIA patients and Tfh cells derived from the tonsils of control individuals. While SF PD-1^{high}CD4+ T cells induced plasmablast differentiation and Ig secretion that was as potent as classic tonsil Tfh cells, the SF PD-1^{high} T cells, but not tonsil Tfh cells, skewed B cell differentiation toward a CD21$^{low/-}$CD11c+ phenotype (Figures 6A–D and Supplementary Figure 10, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

Blocking IFNγ, and particularly IL-21, impaired plasmablast differentiation induced by SF PD-1^{high} T cells (Figure 6E), whereas blocking each cytokine alone did not significantly impair CD21$^{low/-}$CD11c+ B cell differentiation (Figure 6F). However, a strategy of co–cytokine blockade, with blocking of IFNγ together with IL-21, partially impaired the induction of CD21$^{low/-}$CD11c+ B cells by SF PD-1^{high} T cells (Figure 6F). Additionally, inhibiting JAK/STAT signaling with ruxolitinib completely blocked plasmablast differentiation and effectively impaired, but did not totally abrogate, the SF PD-1^{high} T cell–induced differentiation of CD21$^{low/-}$CD11c+B cells in vitro (Figure 6F).

Since T-bet has been characterized as one of the defining transcription factors of CD21$^{low/-}$CD11c+ B cells, we additionally analyzed whether SF PD-1^{high}CD4+ T cells would also induce T-bet expression in B cells in vitro. Indeed, SF PD-1^{high}CD4+ T cells induced T-bet expression in control B cells with levels that tended to be higher than those obtained using coculture with tonsil Tfh cells (Supplementary Figure 11, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract).

Paralleling this observation and in accordance with the different cytokine profiles observed in the SF and tonsil PD-1^{high}CD4+ T cells, the addition of IFNγ, but not IL-21 alone, significantly induced in vitro T-bet expression in CD40L-stimulated control B cells (Supplementary Figure 12, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41913/abstract). However, adding IL-21 had a synergistic effect on T-bet expression in IFNγ/CD40L-stimulated B cells (Supplementary Figure 12). Hence, the SF PD-1^{high}CD4+ T cell subset exerts a potent B helper function, not only inducing plasma cell differentiation, but also particularly skewing B cell differentiation toward a T-bet–expressing CD21$^{low/-}$CD11c+ phenotype, partly through the influence of IL-21 and IFNγ.

DISCUSSION

Synovial inflammation in ANA-positive JIA is characterized by B cell hyperactivity; however, the mechanisms driving aberrant B cell activation at the site of inflammation are not yet understood (3,4,6,7). We have previously demonstrated that the SF CD4+ T helper cell pool in ANA-positive JIA patients is particularly enriched in IL-21–secreting cells (21). We have also shown that CD21$^{low/-}$CD11c+ DN B cells accumulate in the joints of these patients (5). In the present study, we demonstrate that the expansion of IL-21–secreting cells is attributable to a distinct subset of PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells. This CD4+ T helper cell subset exerts a potent B helper function and particularly skews B cell differentiation toward a CD21$^{low/-}$CD11c+ phenotype in vitro, suggesting a functional relationship between both cell subsets.

High expression of activation and proliferation markers, as well as contraction of the *TCRVB* repertoire within SF PD-1^{high}CXCR5–HLA–DR+CD4+ T cells, indicates clonal expansion of this T cell subset within the joints of JIA patients.

Furthermore, the increased clonal overlap of PD-1^{high}CXCR5–HLA–DR+CD4+ T cells between different joint sites in individual patients and between different JIA patients, as well as the preferential accumulation in the joints of ANA-positive JIA patients, may also suggest that clonal expansion has been elicited after encountering currently unknown autoantigens/antigens that are present at the site of inflammation. Therefore, PD-1^{high}CXCR5–HLA–DR+CD4+ T cells appear to represent a pathogenic T cell subset that is expanded in a subgroup of JIA patients, particularly in ANA-positive patients. Dysregulation of T helper cells has been observed in the PB of JIA patients (29,30). A circulating subset of "pathogenic-like" CD4+ T helper cells has been detected in the PB of JIA patients that partially resembles the PD-1^{high}CXCR5–HLA–DR+CD4+ T cell subset described in the present report (29). However, whereas these circulating CD4+ T helper cells displayed an inflammatory cytokine pattern, including increased expression of IFNγ, TNF, and IL-17, the PD-1^{high}CXCR5–HLA–DR+CD4+ T cell subset in the SF of JIA patients in the present study also showed increased expression of IFNγ and TNF, but did not show an increase in IL-17 levels. Furthermore, high PD-1 expression on CD4+ T cells in the SF of JIA patients was particularly correlated with increased expression of IL-21.

Hence, high PD-1 expression on SF CD4+ T helper cells seems to represent a surrogate marker of a clonally expanded T helper cell subset that is characterized by IL-21 expression, thereby suggesting a B helper function. This T helper cell subset appears to be functionally distinct from other proinflammatory T helper cell subsets that have previously been characterized in the joints of JIA patients, e.g., Th1, Th17, and Th1/Th17 cells (24,26). However, CD161 expression on a majority of these IL-21+IFNγ+IL-17–PD-1^{high} CD4+ T cells suggests that these cells may have originated from the Th17 subset of T helper cells, as has been described previously as the potential origin of nonclassic Th1 cells (24–26,31–33). IL-12, which, in an environment of inflammation, has been shown to induce a shift from Th17 to Th1/Th17 or nonclassic Th1 cells and has been shown to induce IL-21 and IFNγ–coexpressing T helper cells in vitro, might be one of the factors involved in the conversion of Th17 cells to IL-21+ IFNγ+IL-17– PD-1^{high} CD4+ T cells (7,24–26).

The PD-1^{high}CXCR5–HLA–DR+CD4+ T cells in the joints of JIA patients shared a distinct phenotype with other IL-21–secreting CD4+ T helper cell subsets detected within the inflamed tissue in patients with different autoimmune diseases (20,34). In particular, expression of activation markers (PD-1, OX40, HLA–DR) and a high BLIMP-1:Bcl-6 ratio in conjunction with c-MAF expression, as well as an extrafollicular/inflammatory chemokine receptor pattern (CCR2+CXCR5–), also characterize the transcriptional program of synovial Tph cells first described in patients with seropositive RA (20). Since then, using PD-1^{high}CXCR5– as the common denominator in the phenotype of Tph cells, a potential circulating counterpart of this subset has been detected in the PB of patients with systemic lupus erythematosus and type 1 diabetes and is thought to contribute to disease pathogenesis by inducing autoantibody/antibody-secreting plasma cells (35,36). Indeed, synovial Tph cells in seropositive RA displayed a potent B helper function, as assessed by the capacity to induce plasma cell differentiation and Ig secretion in vitro (20).

Consistent with those studies, we also provide the first evidence of a potent B helper cell function of PD-1^{high}CXCR5–HLA–DR+ T helper cells derived from the SF of ANA-positive JIA patients. Extending the previous work on Tph cells, our current analysis establishes a more differentiated functional impact of Tph cells on B cell differentiation. Indeed, the results we have presented from in vitro experiments and ex vivo studies highly suggest that SF PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells not only induce plasma cell differentiation and Ig secretion but also particularly skew B cell differentiation toward CD21$^{low/-}$CD11c+DN B cells. We assume that the cellular pattern observed in the SF of JIA patients mirrors that in the synovia. However, we were not able to extend our analysis to include synovial tissue from JIA patients, since synovectomy is rarely performed in JIA and ethical considerations prevented collecting synovial biopsy specimens from the children included in the analysis.

CD4+ T helper cells coexpressing IL-21 and IFNγ have been detected in several infections and autoimmune diseases, according to the corresponding setting and cellular phenotype: "Th1-Tfh cells," Tfh-like cells or, as discussed above, Tph cells (18,19,37–44). Concomitantly, differentiation of CD21$^{low/-}$CD11c+ DN B cells appeared to depend on IFNγ and IL-21, and expansion of Th1-Tfh cells correlates with the presence of CD21$^{low/-}$CD11c+ DN B cells (10,41,45).

Consistent with the results from these studies, several findings from our experiments also suggest that Tph cells secreting IL-21 in conjunction with IFNγ support the differentiation of CD21$^{low/-}$CD11c+ DN B cells in the joints of JIA patients. These findings include 1) the correlation between numbers of CD21$^{low/-}$CD11c+ DN B cells and numbers of IL-21 and IFNγ–coexpressing PD-1^{high}CD4+ T cells in the SF of JIA patients, 2) the need for co–cytokine blockade with blocking of IFNγ together with IL-21 to impair SF PD-1^{high}CD4+ T cell–induced B cell differentiation toward CD21$^{low/-}$CD11c+ B cells in vitro, 3) the lack of in vitro induction of CD21$^{low/-}$CD11c+ B cells by tonsil Tfh cells that predominantly express IL-21 and IL-4 but do not show increased expression of IFNγ, and 4) the synergistic effect of IFNγ and IL-21 on T-bet induction in CD40L-stimulated control B cells in vitro. However, whereas plasmablast/plasma cell differentiation in vitro was essentially dependent on JAK/STAT signaling, and in particular was dependent on the expression of IL-21, the mechanisms underlying the induction of CD21$^{low/-}$CD11c+ B cells by SF Tph cells seem to be more complex, since blocking JAK/STAT signaling (thereby blocking the effects of IFNγ and IL-21) only partially impaired differentiation in vitro.

Of note, another essential prerequisite for the formation of CD21$^{low/-}$CD11c+DN B cells is the engagement of B cell–intrinsic

Toll-like receptors (TLRs), including TLR-7 and TLR-9, which sense components of nuclear antigens that are delivered via antinuclear B cell receptors (BCRs) (10,12,13,46). Although this process could not be addressed in the present study, it is tempting to speculate that expansion of CD21$^{low/-}$CD11c+ DN B cells in the joints of ANA-positive JIA patients might have been induced after concomitant signaling through antinuclear BCRs and TLR-7/TLR-9 in the presence of IL-21 and IFNγ secreted by Tph cells (10,47).

In summary, we have characterized a distinct subset of PD-1^{high}CXCR5–HLA–DR+ CD4+ T cells that is clonally expanded in the joints of ANA-positive JIA patients and that displays a phenotype similar to that of Tph cells. These Tph cells functionally differ from classic Tfh cells and particularly promote differentiation of CD21$^{low/-}$CD11c+ DN B cells that are concomitantly expanded in the joints of ANA-positive JIA patients. These cells are potentially triggered by autoantigens/antigens present at the site of inflammation. Hence, the characteristic expansion of Tph cells and CD21$^{low/-}$CD11c+ DN B cells in the joints of ANA-positive JIA patients might reflect the autoimmune response at the site of inflammation.

ACKNOWLEDGMENT

We would like to thank the FACS Core Unit of the IZKF Würzburg for supporting this study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Morbach had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Fischer, Dirks, Girschick, Morbach.

Acquisition of data. Fischer, Dirks, Klaussner, Haase, Holl-Wieden, Hofmann, Hackenberg, Girschick, Morbach.

Analysis and interpretation of data. Fischer, Dirks, Klaussner, Haase, Hofmann, Hackenberg, Girschick, Morbach.

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