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Next-Generation Sequencing Reveals Restriction and Clonotypic Expansion of Treg Cells in Juvenile Idiopathic Arthritis

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Objective. **Treg cell–mediated suppression of Teff cells is impaired in juvenile idiopathic arthritis (JIA); however, the basis for this dysfunction is incompletely understood. Animal models of autoimmunity and immunodeficiency demonstrate that a diverse Treg cell repertoire is essential to maintain Treg cell function. The present study was undertaken to investigate the Treg and Teff cell repertoires in JIA.**

Methods. **Treg cells (CD4+CD25+CD127^{low}) and Teff cells (CD4+CD25−) were isolated from peripheral blood and synovial fluid obtained from JIA patients, healthy controls, and children with Lyme arthritis. Treg cell function was measured in suppressive assays. The T cell receptor β chain (*TRB*) was amplified by multiplex polymerase chain reaction and next-generation sequencing was performed, with amplicons sequenced using an Illumina HiSeq platform. Data were analyzed using ImmunoSEQ, International ImMunoGeneTics system, and the Immunoglobulin Analysis Tools.**

Results. **Compared to findings in controls, the JIA peripheral blood Treg cell repertoire was restricted, and clonotypic expansions were found in both blood and synovial fluid Treg cells. Skewed usage and pairing of *TRB* variable and joining genes, including overuse of gene segments that have been associated with other autoimmune conditions, was observed. JIA patients shared a substantial portion of synovial fluid Treg cell clonotypes that were private to JIA and not identified in Lyme arthritis.**

Conclusion. **We identified restriction and clonotypic expansions in the JIA Treg cell repertoire with sharing of Treg cell clonotypes across patients. These findings suggest that abnormalities in the Treg cell repertoire, possibly engendered by shared antigenic triggers, may contribute to disease pathogenesis in JIA.**

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease affecting the pediatric population, yet its cause is unknown (1). The importance of T lymphocytes in the pathogenesis of the oligoarticular and polyarticular forms of the disease is evidenced by the demonstration of HLA- and other T cell–related genetic associations, accumulation of activated T cells in JIA synovial fluid (SF), and efficacy of T cell–targeted therapies (2–6). Notably, studies of JIA SF have identified

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T cells with skewed complementarity-determining region 3 (CDR3) length distributions and T cell receptor β chain variable (*TRBV*) family expression (7–9). These findings suggest the existence of clonally expanded T cells in JIA SF.

Treg cells, a subset of T lymphocytes, are important in dampening the autoimmune response in inflammatory arthritis (10). In JIA, the degree of Treg cell enrichment in the SF is inversely correlated with both disease severity and the number of Th17 cells in the joint (11,12). SF Treg cells from patients with JIA are unable to inhibit autologous SF Teff cells (13,14). The cause of this breach in immune tolerance in the arthritic joint is not fully understood. Recent studies have focused on the role of inflammatory cytokines in promoting Teff cell resistance to Treg cell–mediated control (15,16). However, the competence of the Treg cell population is driven also by its specific T cell receptor (TCR) repertoire. Several lines of evidence indicate that a diverse Treg cell repertoire is essential for protection against autoimmunity. Treg cell repertoire skewing has been documented in mouse models of diabetes and arthritis, and may contribute to autoimmune diathesis in these animals (17–19). Adoptive transfer of polyclonal Treg cells ameliorates arthritis in several mouse models (10,20). In human autoimmune conditions, the characteristics of the Treg and Teff cell repertoires have not been evaluated.

The most common form of the TCR is composed of α- and β-chains, which are encoded by distinct loci (*TRA*, *TRB*). The *TRB* locus contains a higher number of coding V, D, and J elements than the *TRA* locus, and therefore, a higher degree of heterogeneity may be observed in the TCRβ (TRB) repertoire. Accordingly, we analyzed the TRB repertoires of Treg and Teff cells from patients with JIA. The analysis was performed using next-generation sequencing (NGS), which offers multiple advantages compared to traditional methods used to study the lymphocyte repertoire, such as CDR3 spectratyping and flow cytometric analysis of Vβ family expression. Previously, it was difficult to sequence large numbers of TCRs, and thus identification of T cell clones and quantification of the true diversity of the T cell repertoire were challenging. In contrast, NGS uses massive parallel sequencing to process millions of rearranged TCR products simultaneously, allowing in-depth analysis of individual TCRs at a nucleotide level while expanding coverage of the total lymphocyte repertoire.

Using NGS, we identified alterations in the TRB repertoires of JIA Treg cells that were not restricted only to the SF, but also found in peripheral blood (PB). Importantly, JIA PB and SF Treg cells manifested oligoclonal expansions, and multiple SF Treg cell *TRB* clonotypes were shared among JIA patients. These findings provide insight into the characteristics of the Treg cell repertoire in JIA and suggest that Treg cell restriction and clonotypic expansion may contribute to disease pathogenesis.

PATIENTS AND METHODS

Study subjects. We performed a cross-sectional and comparative analysis of the TRB repertoires of Treg and Teff cells from the PB and SF of patients with JIA as defined according to the criteria of the International League of Associations for Rheumatology (21). PB and SF samples were obtained during therapeutic joint aspiration. Patients who had received an intraarticular steroid injection in the same joint within the preceding 6 months were excluded. SF samples from children with Lyme arthritis, diagnosed based on positive enzyme-linked immunosorbent assay and Western blot results, were studied as inflammation controls. PB was obtained from healthy controls seen in the rheumatology clinic for joint pain with a noninflammatory cause or in the primary care clinic for routine well child care. Clinical data were acquired from medical records. This study was performed in accordance with the Boston Children's Hospital Institutional Review Board, with informed consent obtained from participants/legal guardians.

Cell isolation and intracellular staining. Mononuclear cells from PB and SF were isolated by Ficoll-Hypaque density-gradient centrifugation (GE Healthcare). CD4+ T cells were enriched from the mononuclear cells by negative selection using magnetic beads (Miltenyi Biotec) and stained with antibodies: fluorescein isothiocyanate–conjugated CD4 (BD Biosciences), phycoerythrin (PE)–conjugated CD25 (eBiosciences), and PE-Cy5– or PE-Cy7–conjugated CD127 (eBiosciences). Fluorescence-activated cell sorting (FACS) (Aria II; BD Biosciences) was used to isolate Treg cells ($CD4+CD25+CD127^{low}$) and Teff cells (CD4+CD25–).

To confirm purity, part of the sample was fixed, permeabilized (eBiosciences), and stained with allophycocyanin-conjugated anti-FoxP3 antibody (eBiosciences). Expression of FoxP3 in the sorted Treg and Teff cell populations was verified by flow cytometry (BD LSRFortessa; BD Biosciences).

Suppression assays. The suppressive capacity of isolated Treg cells was tested against PB Teff cells from a common third-party donor. FACS-isolated Teff cells were labeled with CellTrace Violet (Life Technologies), stimulated with anti-CD2/CD3/CD28 beads (Miltenyi Biotec), and cocultured with Treg cells at a ratio of 1:1. After 4 days, proliferation was measured by CellTrace Violet dye dilution as assessed by flow cytometry (BD LSRFortessa).

T cell repertoire analysis. DNA was extracted from the sorted lymphocyte populations using a Qiagen DNA Mini kit. Multiplex polymerase chain reaction (PCR) was used to amplify the rearranged CDR3β, using a standard quantity of DNA as the template (Adaptive Biotechnologies) (22). PCR products were sequenced using the Illumina HiSeq platform. The sequences were aligned to a reference genome, and *TRB* variable, diversity, and joining (*V*[D]*J*) gene definitions were based on the international ImMunoGeneTics (IMGT) system (23). Correction for PCR amplification bias was previously

determined by using a complete, synthetic repertoire of TCRs to establish an amplification baseline and adjust the assay chemistry to minimize primer bias. Residual PCR bias was addressed by using barcoded, spiked-in synthetic templates to assess sequence coverage. Resulting data were filtered and clustered using the relative frequency ratio between similar clones and a modified nearest neighbor algorithm to merge closely related sequences and remove both PCR and sequencing errors, as previously described (22,24).

Subsequently, productive sequences were analyzed; repertoire clonality and diversity measures along with TCR *V*(D)*J* usage and the average index of CDR3 hydrophobicity (measured with the Kyte-Doolittle scale) were determined using the ImmunoSEQ set of online tools, the IMGT HighV-Quest platform, and the Immunoglobulin Analysis Tool (23,25). Specifically, repertoire diversity was measured by Shannon entropy while clonality was measured by evaluating the frequency of the most abundant TRB clonotypes as well as determining the clonality index. The clonality index is the inverse of Pielou's evenness index J (J = H/log[S]), where H is the Shannon entropy index and S is the number of unique productive sequences (26,27). Rarefaction curves were generated with the PAST program (28).

HLA typing. High-resolution typing of HLA–DRB1, DRB3/4/5, DQA1, DQB1, DPA1, and DPB1 was performed by Sanger sequencing–based typing. Direct genomic DNA sequencing was performed on a 3730XL Genetic Analyzer (Applied Biosystems) for exon 2 of all HLA class II loci, and for exon 3 when necessary to resolve ambiguities. Data were analyzed using uType Sequencing Analysis Software (Life Technologies).

Statistical analysis. Depending on the normality of data, values are expressed as the mean ± SEM or the median and interquartile range (IQR). Student's *t*-tests or Mann-Whitney tests were used to assess the significance of differences between groups. Shannon entropy in paired PB and SF samples from JIA patients was evaluated by paired nonparametric analysis of variance (ANOVA) with Dunn's correction for multiple comparisons. TRB clonality of T cell subsets was compared by one-way ANOVA with Bonferroni adjustment for multiple comparisons. The median difference between shared TRB clonotypes defined by amino acid and *V–J* pairing in SF versus PB Treg cells and in SF versus PB Teff cells was assessed by Wilcoxon matched pairs signed rank test. Two-way ANOVA with Bonferroni adjustment was used to compare *TRBV* family and *TRBJ* gene usage. Venn diagrams were created with Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The *V–J* pairing head maps were created with GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). Analyses were performed with GraphPad Prism version 6.0.

RESULTS

Paired PB and SF samples were obtained from 8 patients with JIA, and SF samples alone from an additional 3. Control samples included PB from 3 healthy children and SF from 2 children with Lyme arthritis (Table 1). As reported previously (12), Treg cells were enriched in JIA SF (median percent CD4+ lymphocytes 15.0 [IQR 12.0–17.7]) compared to Lyme arthritis SF (median 7.4 [IQR 4.2–10.6]), JIA PB (median 5.5 [IQR 4.5–7.6]), and healthy control PB (median 6.9 [IQR 6.5–8.7]) (see Supplementary Figure 1A, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract). FoxP3 expression was confirmed in the FACS-sorted CD4+CD25+CD127^{low} cells (Supplementary Figure 1B). All JIA SF Treg cell samples that were tested (n = 5) were able to inhibit proliferation of PB Teff cells from a third-party control (Supplementary Figure 1C). NGS of the *TRB* rearranged products was completed successfully in all samples except for PB Treg cells obtained from 1 JIA patient (Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract). The primary NGS data are available at www.adaptivebiotech.com/pub/Henderson-2015.

Table 1. Characteristics of the study subjects*

Subject	Age (years)/ sex	JIA subtype	Disease duration, years	No. of active joints	Current treatment
JIA1	8.8/F	Oligo	6	1	MTX
JIA2	9.3/F	Oligo	7	2	LEF
JIA3	12.3/M	Oligo	0.2	2	NSAIDs
JIA4	8.8/F	Psoriatic	1	3	LEF, ETN
JIA5	7.5/M	Oligo	3	3	NSAIDs
JIA6	13.6/F	Oligo	9	4	MTX
JIA7	4.9/F	Oligo	3	2	NSAIDs
JIA8	10.3/F	Oligo	0.2	1	NSAIDs
JIA9	15.1/F	Oligo	0.1	4	NSAIDs
JIA10	11.3/F	Oligo	0.3	1	NSAIDs
JIA11	8.0/F	Oligo	2	1	None
Lyme1	13.8/M	–	0.3	1	NSAIDs†
Lyme2	10.8/M	–	0.3	1	CTX, NSAIDs†
HC1	13.1/F	–	–	–	–
HC2	16.1/M	–	–	–	–
HC3	9.2/M	–	–	–	–

* JIA = juvenile idiopathic arthritis; Oligo = oligoarticular; MTX = methotrexate; LEF = leflunomide; NSAIDs = nonsteroidal anti-inflammatory drugs; ETN = etanercept; Lyme = Lyme arthritis; CTX = ceftriaxone; HC = healthy control.
† A 2-month course of doxycycline treatment had been recently completed.

Restricted TRB repertoire in JIA PB Treg cells. Shannon entropy, which reflects the number of unique TCR sequences and the relative proportion of these sequences in a sample, was used to assess TRB repertoire diversity. As described previously (29–31), the Treg and Teff cell repertoires in control PB samples were equally diverse. In contrast, the JIA PB Treg cell repertoire was restricted compared to those of control PB Treg cells ($P = 0.02$) and JIA PB Teff cells ($P < 0.01$) (Figure 1A). While there was a trend toward reduced diversity in JIA SF Treg and JIA SF Teff cells compared to JIA PB Treg and JIA PB Teff cells, respectively, these differences did not reach statistical significance.

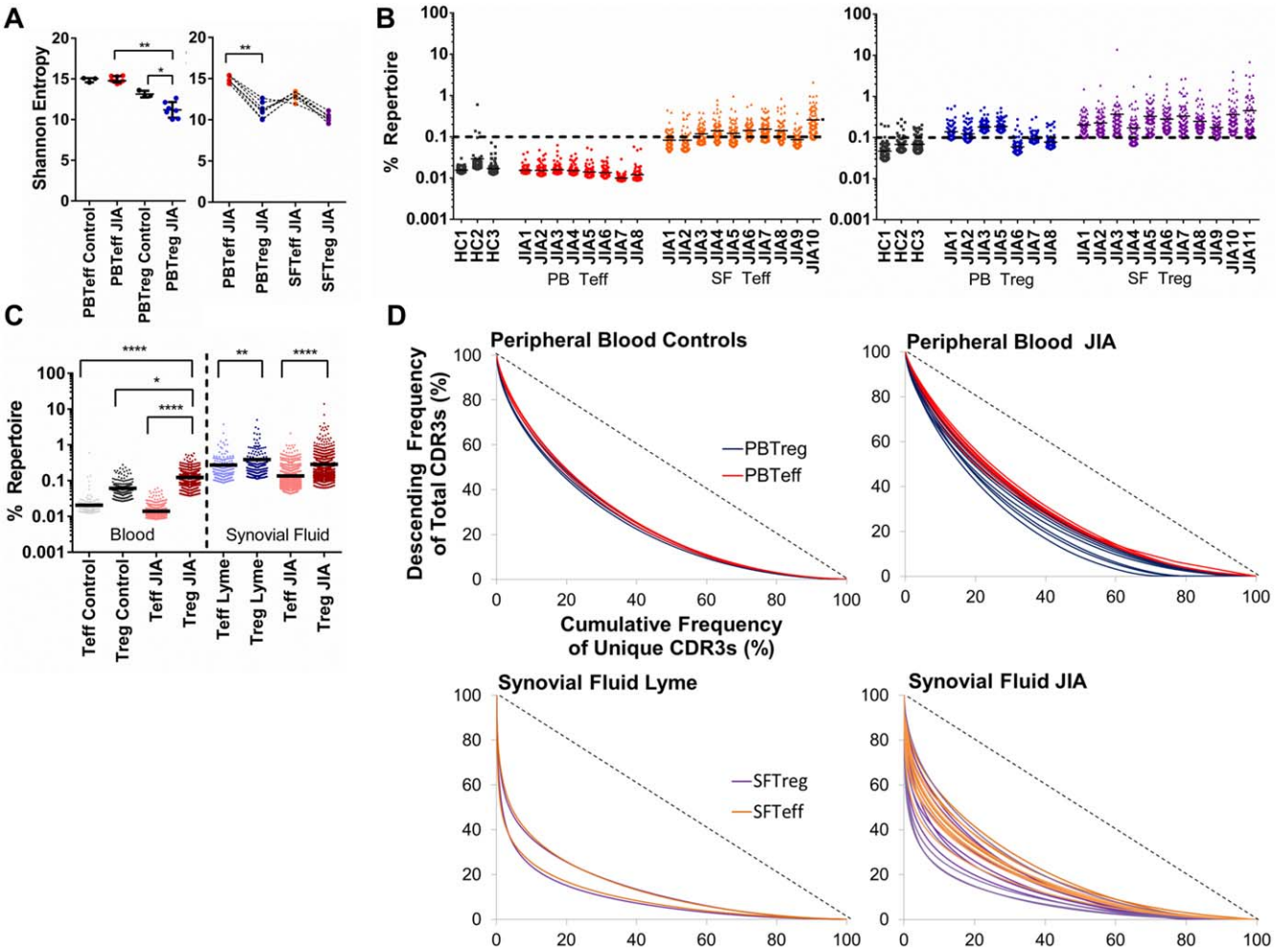

Figure 1. The T cell receptor β (TRB) repertoire of Treg cells from patients with juvenile idiopathic arthritis (JIA) is restricted with clonotypic expansions. **A,** Shannon entropy was plotted for each sample. Each symbol represents an individual subject; bars (left panel) show the median and interquartile range. The Mann-Whitney test (left panel) and paired, nonparametric analysis of variance (ANOVA) with Dunn's correction for multiple comparisons (right panel) were used to evaluate repertoire diversity. $* = P < 0.05$; $** = P < 0.01$. **B,** The 100 most abundant TRB clonotypes from each sample were plotted, and the average frequency is depicted. Each dot represents the frequency of a TRB clonotype (defined by amino acid sequence and *V–J* pairing), expressed as a percentage of all productive sequences obtained from a given sample. In control peripheral blood (PB) Treg and Teff cell samples, 95% of the TRB clonotypes occurred at a frequency of <0.1% (dashed lines). **C**, The "top 100" clonotypes from each sample were then pooled and plotted for each T cell subset. The average frequency of the TRB clonotypes in each group was compared by ANOVA with Bonferroni adjustment for multiple comparisons ($* = P < 0.05$; $** = P < 0.01$; $**** = P < 0.0001$). Horizontal lines in **B** and **C** show the median. **D,** The frequency of unique third complementarity-determining regions (CDR3s), defined by nucleotide use, versus the descending frequency of total CDR3s was plotted. A sample with an even distribution of TRB clonotypes would be represented by the dashed line at 45°. Deviations from this line indicate clonotypic expansions. SF = synovial fluid; HC = healthy control; Lyme = Lyme arthritis.

Clonotypic expansions in the JIA PB Treg repertoire. Clonally expanded populations of T cells were studied by selecting the 100 most abundant TRB clonotypes, defined by amino acid sequence and *V–J* pairing, in each sample. These "top 100" clonotypes were pooled by subject group (JIA, control, or Lyme arthritis) and T cell subset (PB Treg, PB Teff, SF Treg, or SF Teff cells), and the average frequency of the TRB clonotypes in each group was compared. As reported in adult controls (29–31), the Treg and Teff repertoires in the PBs of healthy children were equally polyclonal. The TRB repertoire of PB Treg cells from patients with JIA was significantly more clonal than those of control PB Treg, control PB Teff, and JIA PB Teff cells ($P < 0.0001$) (Figures 1B and C). Interestingly, these repertoire abnormalities in the PB of JIA patients were restricted

to Treg cells, as JIA PB Teff cells remained polyclonal and were similar to control PB Teff cells (Figures 1B and C). These findings were observed not only in the 100 most abundant clonotypes of each sample but also across the entire repertoire, as demonstrated by the cumulative clonotype graphs shown in Figure 1D.

Dominant clonotypes found in the JIA SF Treg repertoire. In SF from the inflamed joints of patients with JIA, large clonotypic expansions were noted in Treg and Teff cells. The TRB repertoire of JIA SF Treg cells was significantly more clonal than the repertoires of JIA PB Treg and Teff cells, JIA SF Teff cells, and control PB Treg and Teff cells ($P < 0.0001$) (Figures 1B and C). The most expanded SF Treg cell clonotype in a JIA patient accounted for 14.0% of that individual's SF Treg cell repertoire, while the largest JIA SF Teff cell clonotype represented 2.1% of the SF Teff cell repertoire (Figure 1B). These dominant SF Treg cell clonotypes, representing >1% of the SF Treg cell repertoire, were found in almost all JIA patients (10 of 11) (Figure 1B). Similar to the findings in patients with JIA, the Lyme arthritis SF Treg cell repertoire was more clonal than the Lyme arthritis SF Teff cell repertoire (Figure 1C). These findings were confirmed in cumulative clonotype graphs representing the clonality of the entire TRB repertoire of each sample (Figure 1D).

Factors associated with JIA SF Treg clonality. To further evaluate factors associated with SF Treg cell clonotypic expansion in patients with JIA, the clonality index, a measure of repertoire evenness that is normalized to the total number of sequence reads, was determined for each sample. The clonality index ranges from 0 to 1, with a value of 1 representing a completely clonal repertoire. The degree of Treg and Teff cell clonality in the SF of JIA patients did not correlate with disease duration or age. There was a trend toward higher Treg clonality in the SF of patients treated with nonsteroidal antiinflammatory drugs alone (median clonality index 0.22 [IQR 0.14–0.25]) compared to patients taking disease-modifying antirheumatic drugs or biologic agents (median 0.12 [IQR 0.10–0.15]) ($P = 0.11$).

Intraindividual *TRB* clonotype sharing among JIA SF Treg, SF Teff, and PB Treg cell populations. In individual JIA patients, the SF Treg cell repertoire shared the greatest proportion of unique *TRB* clonotypes with the SF Teff cell repertoire (median % shared clonotypes 15.5 [IQR 12.6–21.7]) and the PB Treg cell repertoire (median % shared clonotypes 6.2 [IQR 3.6–7.8]) (see Supplementary Figure 2, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract), suggesting that JIA SF Treg cell populations are derived from both thymus-derived and peripherally derived Treg cells. There was also appreciable sharing of SF and PB Teff cell populations in patients with JIA. In contrast, PB Treg and Teff cell repertoires were minimally related, both in control subjects and in JIA patients (Supplementary Figure 2).

Interindividual *TRB* clonotype sharing is preferentially observed in JIA SF Treg cell populations. We postulated that JIA patients may share SF *TRB* clonotypes that recognize the same (auto)antigen(s). To test this hypothesis, the percentage of shared total *TRB* clonotypes, defined by nucleotide sequence in the CDR3, was determined in subject pairs. A minimal degree of clonotype sharing was observed in JIA and control PB Teff cells (Figure 2A), and <1% repertoire overlap was detected in PB Treg cell populations (Figure 2B). Modest clonotype sharing was found in JIA SF Teff cells (Figure 2C). Notably, significant sharing (accounting for up to 19% of all clonotypes) was demonstrated in SF Treg cells from JIA patient pairs (Figure 2D and Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract). This large degree of repertoire overlap in JIA SF Treg cells was due to sharing of clonotypes that were expanded (mean ± SEM copy number of shared JIA SF Treg cell clonotypes and shared JIA SF Teff cell clonotypes $1{,}068.0 \pm 512.6$ and 341.9 ± 68.8, respectively; $P = 0.006$). Interestingly, these shared SF Treg cell clonotypes were private to JIA and were not found in children with Lyme arthritis (Figure 2D), suggesting preferential occurrence of disease-specific *TRB* clonotypes in JIA SF.

HLA typing and JIA SF Treg cell clonotype sharing. HLA is an important genetic factor that shapes the TCR repertoire. Known HLA class II associations in oligoarticular JIA include HLA–DRB1*0801, 1103, 1104, 1301, and DPB1*0201 (32–34). To determine whether sharing of HLA alleles could explain the observed SF Treg cell repertoire overlap, each subject underwent high-resolution typing of the HLA class II locus (Supplementary Table 3, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract). JIA patients 2 and 4, who were each DRB1*1103/1104 and DPB1*0201 positive, shared a substantial portion of SF Treg clonotypes. JIA patients 4 and 5 shared the DPB1*0201 allele and had SF Treg and Teff cell repertoire overlap. In contrast, JIA patients 2 and 10 shared the DRB1*1301;DQA1*0103;DQB1*0603 predisposing haplotype but did not exhibit substantial repertoire sharing. The repertoire overlap between patient 3 and patients 1, 4, 5, and 7 was not explained by HLA class II typing. Therefore, the observed SF Treg cell clonotype sharing was only partially explained by expression

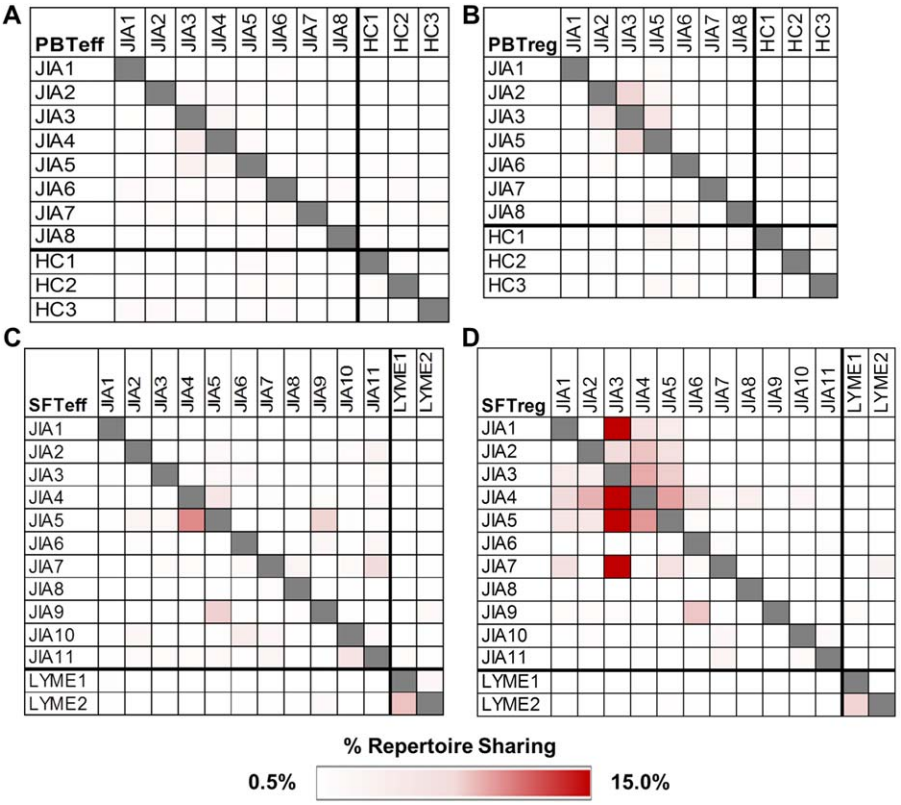

Figure 2. *TRB* clonotypes are preferentially shared in SF Treg cells of JIA patients. The number of shared *TRB* clonotypes between subject pairs was expressed as a percentage of each subject's total productive T cell sequences in **A,** PB Teff cell, **B,** PB Treg cell, **C,** SF Teff cell, and **D,** SF Treg cell populations. A *TRB* clonotype was defined by an identical nucleotide sequence in the CDR3 region. The degree of *TRB* clonotype overlap is depicted by the intensity of the color shading, with white representing minimal commonality and red indicating substantial clonotype sharing. See Figure 1 for definitions.

of common HLA class II alleles, and was likely mediated by other environmental and/or genetic factors.

Convergent recombination in JIA SF Treg cells. Convergent recombination is an indication of antigen-driven immune responses and has been observed in certain infections and autoimmune responses (35). In this process, different *V(*D*)J* gene combinations are used to produce TCRs with the same amino acid sequence in the antigen-binding site (CDR3). To investigate for evidence of a T cell response to a common antigen, we assessed convergent recombination in JIA T cells. Specifically, we compared the frequency of shared clonotypes, defined either by identity of CDR3 amino acid sequence alone or by amino acid sequence and *V–J* gene pairing. Convergent recombination was seen preferentially in the TRBs of SF Treg cells compared to PB Treg cells ($P = 0.005$) (Figures 3A–C). Interestingly, among JIA Teff cells, significantly more convergent recombination was observed in cells from PB compared to SF ($P < 0.0001$) (Figure 3D). These findings further support the hypothesis that shared (auto)reactive clones are contained in the JIA SF Treg repertoire.

Skewed usage and pairing of TRBV and TRBJ genes in JIA. The CDR3 domain of the TCR is encoded by *V(*D*)J* genes. To capture possible skewing of the TCR repertoire at the global population level, we analyzed usage of *Vβ* and *Jβ* family genes among total clonotypes. Compared to cells from healthy controls, JIA PB Treg and Teff cells expressed *TRBV7* significantly more frequently, and *TRBV3* significantly less frequently. Among JIA PB Teff cells, the *TRBJ02–07* gene was used significantly more, whereas *TRBJ01–03* was used significantly less, than in controls. In JIA patients, differential *TRBJ* usage was noted in SF compared to PB T cell populations. Detailed results of these analyses are shown graphically in Supplementary Figure 3, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract.

At the total population level, abnormal *Vβ* and *Jβ* usage in the *TRB* repertoire may reflect preferential

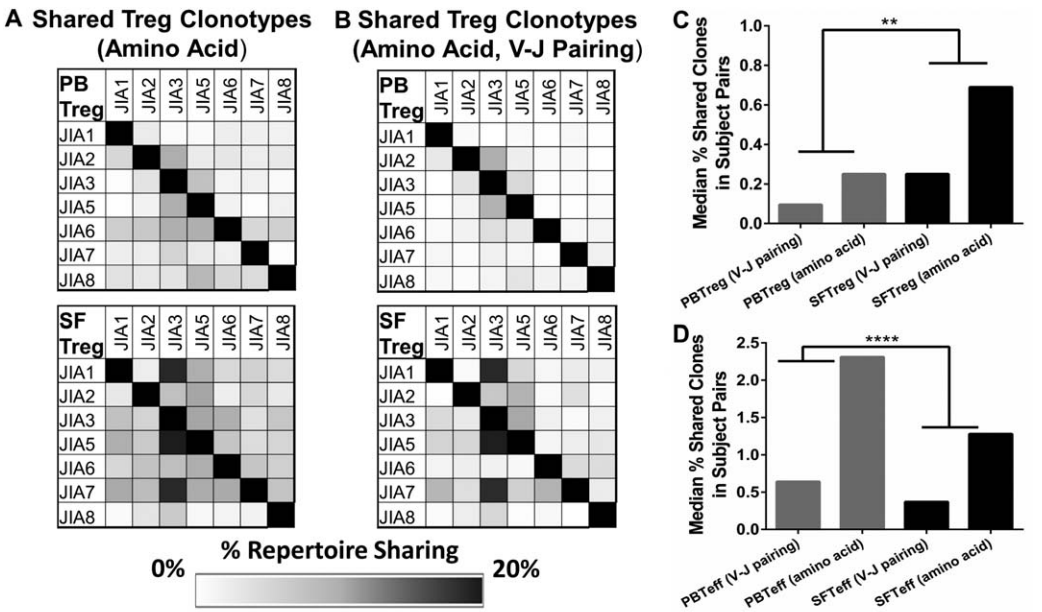

Figure 3. Preferential convergent recombination in SF Treg cells of patients with JIA. The percentage of shared Treg cell TRB clonotypes in PB samples or SF samples from pairs of JIA patients was determined. **A** and **B,** A TRB clonotype was defined by either identical amino acid sequence (**A**) or identical amino acid sequence and *V–J* gene pairing in the CDR3 region (**B**). The degree of TRB clonotype overlap is depicted by the intensity of the color shading, with white representing minimal commonality and black indicating substantial sharing. Subjects with paired PB and SF samples were included in the analysis. **C** and **D,** The median percent of shared TRB clonotypes in Treg cells (**C**) and Teff cells (**D**) from subject pairs was plotted. Increased sharing of TRB clonotypes defined by amino acid sequence compared to clonotypes defined by *V–J* pairing is suggestive of convergent recombination. To evaluate for convergent recombination in JIA, the median difference between shared TRB clonotypes defined by amino acid and *V–J* pairing in SF Treg cells versus PB Treg cells was determined. The same analysis was conducted to compare SF and PB Teff cells. ** = $P = 0.005$; **** = $P < 0.0001$, by Wilcoxon matched pairs signed rank test. See Figure 1 for definitions.

selection of certain *V(*D*)J* genes during T cell generation in the thymus. Alternatively, these differences may be due to expansion and/or survival of T lymphocytes expressing certain *V–J* pairings in the periphery. To investigate this further, we repeated the same analysis on unique sequences and found no differences in *TRBV* or *TRBJ* expression between healthy controls and JIA patients. These findings suggest that the observed *TRBV* and *TRBJ* skewing at the total population level was due to selection in the periphery, possibly in response to common auto- or infectious antigens.

Evaluation of *V–J* gene pairing revealed that the PB Teff cells of JIA patients and healthy controls had a similar pattern of *V–J* gene expression, which was diverse and even (Figure 4). Uniquely, *V–J* gene pairing was more uneven in JIA PB Treg cells compared to control PB Treg cells, with loss of certain *V–J* gene combinations and enrichment of others (Figure 4). These findings corroborate the observed JIA PB Treg cell repertoire restriction and clonotypic expansion. JIA SF Treg cells showed a similarly irregular pattern of *V–J* gene expression. Rarefaction curves confirmed adequate coverage of the JIA SF and PB Treg repertoires (Supplementary Figure 3, http://onlinelibrary.wiley.com/doi/10.1002/art.39606/abstract); thus, these *V–J* pairing abnormalities were not due to insufficient sampling.

Differences in CDR3 hydrophobicity in JIA SF. In B cells, more hydrophobic amino acids in the CDR3s are thought to promote autoimmunity by allowing nonspecific binding of antigens (36,37). Less is known about how CDR3 charge influences the function of human T cells. Whether human Treg and Teff cell populations differ in their CDR3 hydrophobicity profile has not been previously explored. We used the Kyte-Doolittle scale to quantify the mean hydrophobicity of the amino acid residues in the CDR3 (positive values indicate increased hydrophobicity). As previously described for murine Treg cells (38), human Treg cell CDR3 sequences were consistently more hydrophobic than their Teff cell counterparts in the PB of controls (average score on the Kyte-Doolittle scale −0.125 versus −0.136 in control PB Treg cells and Teff cells, respectively; $P < 0.0001$), the PB of JIA patients (−0.120 versus −0.132, respectively; $P < 0.0001$), and the SF of Lyme arthritis patients (−0.135 versus −0.150, respectively; $P < 0.0001$). In contrast, JIA SF

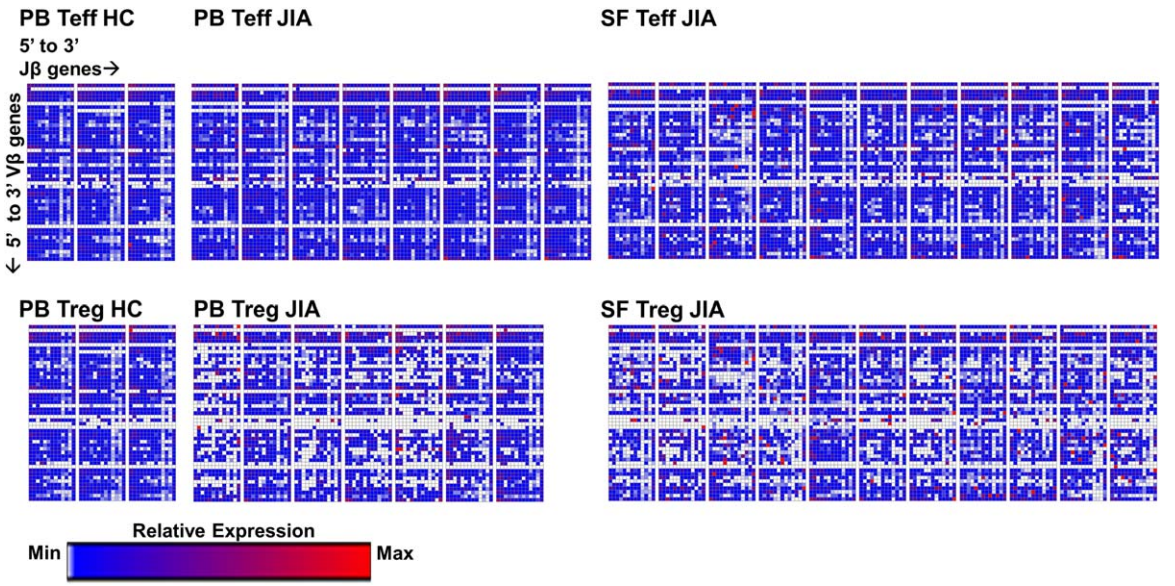

Figure 4. Uneven *Vβ* and *Jβ* pairing in PB and SF Treg cells of patients with JIA. *Vβ* and *Jβ* pairing in total *TRB* clonotypes of PB and SF Treg and Teff cells from patients with JIA as well as PB Treg and Teff cells from healthy children is depicted in the heatmaps. White represents the absence of a given *Vβ* and *Jβ* pairing, blue reflects a low frequency, and red represents a higher frequency of use. See Figure 1 for definitions.

Treg cell populations were not significantly more hydrophobic than JIA SF Teff cells (−0.131 versus −0.133, respectively; $P = 0.11$) (Figures 5A and B). These findings suggest that the Treg cells present in the arthritic joints of JIA patients have unique antigen-binding site features.

DISCUSSION

We describe herein a comparative analysis of the TRB repertoires of Treg and Teff cells in patients with JIA. Striking abnormalities in the TRB repertoire of Treg cells, including alterations in diversity, clonality, TRB clonotype overlap, *Vβ* and *Jβ* gene usage and pairing, and

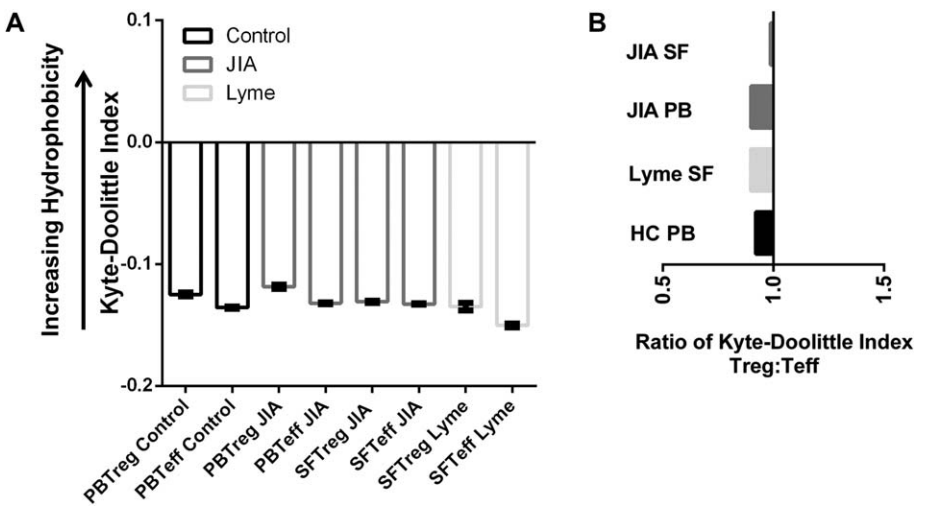

Figure 5. Differential CDR3 hydrophobicity in SF Treg cells of patients with JIA. **A,** Hydrophobicity of the amino acids in the CDR3 of the TRB in PB Treg and Teff cells from healthy children, PB Treg and Teff cells from JIA patients, SF Treg and Teff cells from JIA patients, and SF Treg and Teff cells from children with Lyme arthritis. Values are the mean ± SEM. **B,** Ratio of the mean Kyte-Doolittle hydrophobicity score in Treg cells to the mean score in Teff cells in SF and PB from JIA patients, SF from children with Lyme arthritis, and PB from healthy children. A ratio of <1 indicates that Treg cell clonotypes are more hydrophobic than Teff cell clonotypes; a ratio of >1 indicates that Treg cell clonotypes are less hydrophobic than Teff cell clonotypes. See Figure 1 for definitions.

CDR3 hydrophobicity, were observed in all JIA patients. Subtle skewing in the usage of *TRBV* and *TRBJ* genes in Teff cells was also found. The repertoire anomalies in JIA encompassed Treg and Teff cells in both PB and SF, indicating a profound alteration in TCR repertoire homeostasis.

While Treg cells are a small fraction of the total CD4+ T cell population, their repertoire is disproportionately diverse. The complexity of the human Treg cell repertoire rivals those of Teff (CD4+CD25−) cells and naive CD4+CD45RA+ T cells (29–31). Mouse models demonstrate that Treg cell diversity is important for maintaining immune tolerance. In NOD mice, the Treg cell repertoire is restricted, and Treg cells are unable to respond appropriately in an inflammatory environment (17–19). Transfer of polyclonal Treg cells into NOD mice can reverse early diabetes (39). In SKG mice with mutations in the Zap-70 gene, impaired TCR signaling causes skewed thymic selection of T cells, Treg cell repertoire abnormalities, and impaired Treg cell function, resulting in development of arthritis (18).

Consistent with the results of studies in healthy adults (29–31), PB Treg and PB Teff cells from pediatric control subjects were diverse and polyclonal, with an even pattern of *V–J* pairing. In contrast, the JIA PB Treg cell repertoire was restricted, with uneven *V–J* pairing, and expanded TRB clonotypes were found in JIA PB and SF Treg cell populations. Given the importance of a diverse Treg cell repertoire in preventing autoimmunity in mouse models (10,17,19,20,39), these findings suggest that Treg cell repertoire abnormalities may play a role in the pathogenesis of JIA.

In addition to clonotypic expansion, SF Treg cells from JIA patients exhibited other distinctive features. *TRB* clonotypic sharing was observed more frequently in SF Treg cell compared to SF Teff cell populations; some JIA patients shared up to 19% of the Treg cell repertoire. These shared synovial *TRB* clonotypes tended to be expanded and were private to JIA patients and not found in SF from children with Lyme arthritis. Convergent recombination was also preferentially detected in JIA SF Treg cells. These observations support the notion that there are shared TCR specificities among JIA patients, which are particularly prevalent in SF Treg cells.

Atypical features in the antigen-binding sites of JIA T cells were also noted. Skewed *Vβ* and *Jβ* gene usage and pairing was demonstrated, including increased expression of the *TRBV7* gene segment, which has been associated with other autoimmune conditions (40,41). In addition, a different pattern of *Jβ* gene usage and abnormalities of CDR3 hydrophobicity were detected in JIA SF T cells compared to JIA PB T cell populations. These findings indicate that T cells in patients with JIA have unique CDR3 characteristics, which may influence antigen recognition and T cell responses.

The results of this study raise questions about the role of Treg cell dysfunction in JIA. It is unclear whether TRB clonotypic expansion in SF Treg cells reflects an appropriate but insufficient attempt to control inflammation or a maladaptive response that helps to perpetuate autoimmunity. In this context, the previously described impaired suppressive activity of JIA SF Treg cells (13,14) might reflect repertoire restriction that impedes effective control of Teff cells but might also correlate with pathogenic activity of these expanded clones. It is interesting to note that Treg cells have some degree of plasticity and can adopt Th1- and Th17-like characteristics including production of interferon-γ and interleukin-17 (IL-17) (42–44). In humans, it is unclear whether Treg cells expressing Th1 and Th17 cytokines maintain their suppressive capacity. In murine collagen-induced arthritis, IL-6 promotes the conversion of CD4+FoxP3+ T cells into proinflammatory Th17 cells (45). Additionally, autoantigen-specific Treg cells in experimental autoimmune encephalomyelitis are more likely to lose FoxP3 expression and develop a T effector phenotype, suggesting that antigen-specific Treg cells are more likely to become pathogenic ex-Treg cells (46). In SF from patients with JIA, decreased FoxP3 expression in Treg cells has been documented (47).

The above findings suggest that Treg cells in an inflammatory environment are unstable, may downregulate the FoxP3-dependent transcriptional program, and can evolve to promote inflammation as pathogenic effector T cells. Further study of the cytokine expression and suppressive capacity of clonally expanded JIA SF Treg cells is needed. The stability of Treg cells in the joints of children with arthritis is of particular importance to the potential utility and safety of Treg-immunomodulatory therapies, including IL-2 treatment to expand endogenous Treg cells and adoptive transfer of ex vivo Treg cells.

While providing novel information on the T cell repertoire in JIA, our study has limitations. The majority of the JIA patients studied had the oligoarticular form of the disease, and whether the results can be generalized to other types of JIA remains to be determined. Disease duration varied among the patients, as did the type of immunomodulatory treatment. We are unable to ascertain whether any treatments had an effect on the TCR results; however, the homogeneity of findings across all of the JIA patients indicates that this likely was not the case. Longitudinal study of individual patients will help to determine whether Treg cell repertoire restriction

varies with disease duration and/or treatment. A further limitation arises from the scarcity of available control SF samples from children. We elected to compare JIA SF to SF from children with Lyme arthritis. Persistent arthritis in children with Lyme disease likely represents a reactive autoimmune phenomenon triggered by Lyme disease and not an ongoing infection (48). If this is correct, we would expect that any bias arising from our use of Lyme arthritis patients as a comparator with the JIA patients would be conservative.

The antigen specificity of the clonally expanded SF Treg cell populations remains unknown. Simultaneous analysis of *TRA* and *TRB* rearranged products in single sorted cells may provide definitive evidence of the existence of shared clones that recognize common (self) antigens (49,50). Complementary studies in other forms of arthritis will help to determine whether Treg cell repertoire abnormalities are a common feature of chronic inflammatory arthritis or are unique to JIA.

In summary, NGS analysis revealed reduced Treg cell repertoire diversity in the PB of patients with JIA, suggesting that the competency of the Treg cell compartment may be impaired in affected patients. Our analysis also demonstrated the existence of shared and expanded SF Treg cell clonotypes in JIA, indicating a potentially maladaptive immune response to a common antigen. These observations will serve as the basis for future studies to elucidate the role of Treg cell dysfunction in the pathogenesis and persistence of inflammatory arthritis in children.

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. Henderson 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. Henderson, Volpi, Lee, Nigrovic, Notarangelo.

Acquisition of data. Henderson, Volpi, Frugoni, Janssen, Kim, Sundel, Dedeoglu, Lo, Hazen, Son, Mathieu, Yu, Lebedeva, Fuhlbrigge, Walter, Lee, Nigrovic, Notarangelo.

Analysis and interpretation of data. Henderson, Mathieu, Zurakowski, Yu, Lebedeva, Lee, Nigrovic, Notarangelo.

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