Supplementary Materials and Methods

Samples

EDTA-anticoagulated blood and synovial fluid from 14 polyarticular and extended oligoarticular JIA patients with active disease were collected at the G. Gaslini Institute, Genoa, and IRCCS Policlinico S. Matteo Foundation, Pavia (Italy). Blood from 32 JIA patients before (T0) and after (Tend) at least 6 months of treatment with methotrexate, etanercept and prednisolone (tapered to zero in 4 months) was collected from participants in the TREAT study[34]. Blood from 39 RA patients was collected as part of the DNAJP1 study[36]. Blood from 14 adult healthy controls was collected at the TSRI Normal Blood Donor Service (La Jolla, CA). All samples were collected upon informed consent. All experiments have been conducted according to the principles expressed in the Declaration of Helsinki and IRB approved. Mononuclear cells were separated by density gradient with Histopaque-1077 (Sigma-Aldrich) and frozen in freezing medium (90% FCS, 10% DMSO).

Flow cytometry

Ex vivo immunophenotyping was performed immediately after thawing. Dead cells were excluded using Live/Dead Fixable Near-IR Stain (Life Technologies). Fc receptors (FcR) were blocked using FcR blocking reagent (Miltenyi Biotec) to avoid non-specific antibody binding. Intracellular staining was performed with the FOXP3 buffer set (eBioscience). To measure cytokine production, PBMCs were thawed and allowed to rest overnight before stimulation with 20 nM PMA and 200 nM ionomycin (Fisher Scientific) for 6 hours. GolgiPlug and GolgiStop (BD Biosciences) were added to cell culture 2 hours after PMA/ionomycin stimulation. Samples were acquired with an LSRFortessa (BD Biosciences). Antibodies were from Biolegend, BD Biosciences and eBioscience. Analysis was performed with FlowJo (Treestar). The gating strategy is shown in **Figure S1**. The stability of HLA-DR expression upon PMA/ionomycin stimulation is shown in **Figure S2**.

Cell culture

Complete medium was prepared as follows: RPMI-1640 (HyClone), 10% FBS (Fisher Scientific) for cell lines or 5% heat-inactivated human serum AB (GemCell) for fresh cells, 2 mM L-Glutamine (Gibco) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). T cell lines were established by stimulating freshly sorted HLA-DR⁻CD14⁻CD4⁺ T cells with anti-CD3/CD28-coated beads (Life Technologies) at 1:5 ratio. Complete medium

supplemented with 20 U/ml rhIL-2 (Life Technologies) was added every 2-3 days. After 7 days, dead cells were removed with the Dead Cell Removal kit (Miltenyi Biotec) and viable cells were allowed to rest for 40 hours in complete medium. Before experiments, T cell lines were routinely tested by flow cytometry to confirm that at least 50% of cells were positive for HLA-DR.

Mixed lymphocyte reaction

$CD14^-CD4^+CD25^{low/-}$ T cells were sorted from thawed PBMCs after resting overnight in complete medium with 20 IU/ml rhIL-2. T cells were labeled with Cell Trace Violet (Life Technologies). Allogeneic HLA-DR-expressing T cell lines were labeled with CFDA-SE and used to stimulate sorted T cells at 1:1 ratio in the presence of rhIL-2 (50 IU/ml) and, where indicated, SEB (10 ng/ml, Sigma-Aldrich) to ensure HLA-DR was still expressed during the assay. SEB bridges specific TCR Vβ domains to MHC class II molecules, leading to nonspecific T cell activation[42]. As a control, allogeneic monocyte-derived dendritic cells activated with LPS (50 ng/ml, Sigma-Aldrich) were added to fresh T cells at 1:1 ratio. Dendritic cells were generated by incubating $CD14^+$ monocytes in complete medium with rhGM-CSF (50 ng/ml) and rhIL-4 (10 ng/ml, both cytokines from R&D Systems) for 6 days.

Calcium flux

HLA-DR-expressing T cell lines were loaded with 2 μM Fluo-4 AM (Life Technologies) for 30 min at RT, then placed in Tyrode's solution containing Sytox Red (Life Technologies) as a viability dye. Cells were pre-warmed at 37 degrees before analysis. Calcium flux was measured at 37 degrees for 30 seconds (baseline) + 1 min (addition of mouse primary Ab, 8 μg/ml) + 5 min (addition of anti-mouse IgM/G, 16 μg/ml). Ionomycin was used as a positive control at 2 μg/ml.

Statistical analyses

t-tests were performed for single pairwise comparisons, while ANOVA with post hoc tests was used for multiple comparisons (Tukey's when all possible pairs were assessed, or Dunnett's when each of a number of conditions was compared with a single control). Paired t-tests/repeated measures ANOVA were used in comparisons of matched blood, CPLs and synovium, while unequal variance unpaired t-tests/regular ANOVA were used in all other comparisons.

Hierarchical clustering of phenotypic data was performed using the euclidian distance and Ward linkage. Hierarchical clustering of the TCR repertoire was performed using the Chao-modified Jaccard index[12, 15] and single linkage.

Supplementary References

42.   Li H, Llera A, Malchiodi EL, Mariuzza RA. The structural basis of T cell activation by superantigens. Annual review of immunology. 1999; 17:435-466.

Supplementary figure legends

Supplementary Figure 1. Gating strategy for immunophenotyping of HLA-DR$^+$ T cells. Total PBMCs were thawed and immediately stained with a viability dye, CD14, CD4, CD3, CD25, FOXP3 and HLA-DR, and gated as shown.

Supplementary Figure 2. Stability of HLA-DR upon PMA/ionomycin stimulation. Total PBMCs were allowed to rest overnight in complete medium and then stained with CD4, CD3, CD25, FOXP3 and HLA-DR, either immediately or after 6-hour stimulation with PMA/ionomycin. A representative donor out of 4 is shown.

Supplementary Figure 3. HLA-DR is not endowed with signaling activity in T cells. T cell lines (> 50% HLA-DR$^+$) were obtained by stimulating sorted HLA-DR$^-$CD14$^-$CD4$^+$ T cells from healthy controls for 7 days with anti-CD3/CD28-coated beads. A representative experiment out of 4 is shown. **A.** Calcium flux in Fluo-4-loaded T cell lines. The stimulus (either cross-linking secondary antibody or ionomycin) was added at the time indicated by the arrow. **B.** Antigen-presenting capacity of T cell lines in 5-day co-cultures with fresh autologous T cells labeled with CFSE. In selected conditions, SEB was added as a control that HLA-DR was still expressed during the assay. Allogeneic monocyte-derived dendritic cells activated with LPS were used as a positive control of antigen-presenting cells.

Supplementary Figure 4. CPLs are enriched in clonotypes of synovial T cells. Next-generation sequencing of TCRβ CDR3 sequences was performed on blood or synovial CD4$^+$ T cells of JIA patients with active disease. All data refer to in-frame nucleotide sequences. **A-B.** Renyi diversity indices for different values of α (**A**) and summary at α=1

(**B**; grey dashed line in panel A). Differential species diversity is substantiated only if the Renyi index is consistently greater (or lower) across all values of the α parameter. **C.** Clustering based on TCR repertoire distances, calculated as 1 - Chao-modified Jaccard index (Chao distance). **D.** Summary of the TCR repertoire pairwise Chao distances between CPL or blood samples and their paired synovial sample. **E**. Overlap of the TCR repertoire from CPL and blood samples with their matched synovial sample. Each open circle is the median overlap of 200 subsamples of the indicated size from either CPL or blood sample with 200 subsamples of the same size from their paired synovial sample. **F.** Summary for the overlap data as measured at the size of the smaller sample (grey dashed line in panel E). **G.** Vβ gene family usage. In A, C, E and G, each panel represents an individual patient.