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Pathologically expanded 'peripheral' B cell-helper T cells in rheumatoid arthritis

Deepak A. Rao¹, Michael F. Gurish¹, Jennifer L. Marshall², Kamil Slowikowski^{1,3,4,5,6}, Chamith Fonseka^{1,3,4,6,7}, Yanyan Liu¹, Laura T. Donlin^{8,9}, Lauren A. Henderson¹⁰, Kevin Wei,¹ Fumitaka Mizoguchi¹, Nikola C. Teslovich^{1,3,4}, Michael E. Weinblatt¹, Elena M. Massarotti¹, Jonathan S. Coblyn¹, Simon M. Helfgott¹, Yvonne C. Lee¹, Derrick J. Todd¹, Vivian P. Bykerk^{11,12}, Susan M. Goodman^{11,12}, Alessandra B. Pernis^{9,12,13}, Lionel B. Ivaskiv^{8,9}, Elizabeth W. Karlson¹, Peter A. Nigrovic^{1,10}, Andrew Filer², Christopher D. Buckley², James A. Lederer¹⁴, Soumya Raychaudhuri^{1,3,4,5,15,16}, and Michael B. Brenner¹

1) Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115 USA

2) Rheumatology Research Group, Institute of Inflammation and Ageing, University of Birmingham, Queen Elizabeth Hospital, Birmingham, B15 2WB, UK

3) Division of Genetics, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115 USA

4) Program in Medical and Population Genetics, Broad Institute of Massachusetts Technical Institute and Harvard University, Cambridge, MA 02138, USA

5) Partners Center for Personalized Genetic Medicine, Boston, MA 02115 USA

6) Bioinformatics and Integrative Genomics, Harvard University, Cambridge, MA 02138, USA

7) Biological and Biomedical Sciences, Harvard University, Cambridge, MA 02138, USA

8) Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, New York 10021, USA

9) David Z. Rosensweig Genomics Research Center, Hospital for Special Surgery, New York, New York 10021, USA

10) Division of Immunology, Boston Children's Hospital, Boston, MA 02115 USA

11) Division of Rheumatology, Hospital for Special Surgery, 535 E 70th Street, New York, NY 10021

12) Department of Medicine, Weill Cornell Medical College, Cornell University, New York, NY 10021, USA

13) Autoimmunity and Inflammation Program, Hospital for Special Surgery, New York, NY 10021, USA

14) Department of Surgery, Brigham and Women's Hospital, Boston, MA 02115 USA

15) Reumatology Unit, Karolinska Institutet and Karolinska University Hospital Solna, Stockholm, Sweden

16) Institute of Inflammation and Repair, University of Manchester, Manchester, UK

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SUMMARY

1
2 **CD4⁺ T cells are central mediators of autoimmune pathology; however, the**
3 **definition of their key effector functions in specific autoimmune diseases**
4 **remains limited. Pathogenic CD4⁺ T cells within affected tissues may be**
5 **identified by expression of markers of recent activation¹. We applied this**
6 **approach to joint tissue in rheumatoid arthritis (RA), a chronic immune-**
7 **mediated arthritis that affects up to 1% of the population². Utilizing mass**
8 **cytometry to detect activated T cells in RA synovial tissue revealed a**
9 **strikingly expanded population of PD-1^{hi} CXCR5⁻ CD4⁺ T cells. These cells**
10 **are not exhausted, Rather, multidimensional cytometry, transcriptomics,**
11 **and functional assays define a population of PD-1^{hi} CXCR5⁻ ‘peripheral**
12 **helper’ T (Tph) cells that express factors enabling B cell help, including IL-**
13 **21, CXCL13, ICOS, and MAF. Like PD-1^{hi} CXCR5⁺ T ‘follicular helper’ (Tfh)**
14 **cells, Tph cells induce plasma cell differentiation *in vitro* via IL-21 and**
15 **SLAMF5-interactions^{3,4}. However, global transcriptomics robustly separate**
16 **Tph cells from Tfh cells, with altered expression of Bcl6 and Blimp-1 and**
17 **unique expression of chemokine receptors that direct migration to inflamed**
18 **sites, such as CCR2, CX3CR1, and CCR5, in Tph cells. Tph cells appear**
19 **uniquely poised to promote B cell responses and antibody production**
20 **within pathologically inflamed non-lymphoid tissues.**

21 We analyzed CD4⁺ T cells in 3 seropositive (defined as rheumatoid factor+ or
22 anti-citrullinated peptide antibody+) RA synovial tissue samples with dense
23 leukocyte infiltrates using a mass cytometry panel designed to interrogate both
24 stromal and leukocyte populations (**Extended Data Table 1**). Two-dimensional
25 visualization of the multidimensional cytometry data using the viSNE algorithm⁵
26 revealed a heterogeneous CD4⁺ T cell population with distinct expression
27 patterns of 5 commonly used activation markers (PD-1, MHC II, ICOS, CD69,
28 CD38) (**Fig. 1a**). Strikingly, a large population of cells with high PD-1 expression
29 clustered together in each of the 3 samples (**Fig. 1a, Extended Data Fig. 1a**).
30 Biaxial gating of data from 6 seropositive RA synovial tissue samples confirmed
31 high expression of PD-1 on ~25% of synovial CD4⁺ T cells, the majority of which
32 co-expressed MHC II and/or ICOS (**Fig. 1b, Extended Data Fig. 1b, Extended**
33 **Data Table 2**).

34

35 In a complementary approach, 11-dimensional flow cytometric analysis of
36 memory CD4⁺ T cells from paired synovial fluid and blood samples from 3
37 seropositive RA patients also revealed a large population of synovial PD-1^{hi} CD4⁺
38 T cells, a subset of which co-expressed MHC II and/or ICOS (**Fig. 1c, Extended**
39 **Data Table 3**). Biaxial gating confirmed high PD-1 expression on ~30% of
40 synovial fluid CD4⁺ T cells, mirroring results from synovial tissue (**Fig. 1d,e,**
41 **Extended Data Fig. 1c**). The frequency of PD-1^{hi} CD4⁺ T cell populations in
42 seropositive RA synovial fluid (n=9) was over 5-fold higher than in synovial fluid
43 from 19 patients with seronegative inflammatory arthritides (seronegative RA

44 n=2, spondyloarthropathy n=8, juvenile idiopathic arthritis n=9, $p < 0.0001$, Mann-
45 Whitney) (**Fig. 1d,e**).

46

47 Because seropositive RA is characterized by autoantibody production and
48 frequent synovial T cell-B cell aggregates^{6,7}, we considered whether synovial PD-
49 1^{hi} cells might be Tfh cells. Tfh cells, often identified as CXCR5⁺ PD-1⁺, are
50 uniquely adapted to promote B cell recruitment and differentiation in lymph node
51 follicles via production of IL-21, IL-4, CD40L, and CXCL13, the ligand for
52 CXCR5⁴. However, seropositive RA synovial tissue samples contained few PD-
53 1^{hi} CXCR5⁺ Tfh cells (**Fig. 1f,g**), which clustered separately from PD-1^{hi} CXCR5⁻
54 cells in viSNE analyses (**Fig. 1a**, right panel). In contrast, ~85% of PD-1^{hi} cells in
55 synovial tissue lacked CXCR5, as did almost all PD-1^{hi} cells in synovial fluid (**Fig.**
56 **1f,g**). Measurement of CXCR5 transcript levels in sorted PD-1^{hi} CXCR5⁻ and PD-
57 1^{hi} CXCR5⁺ cells from synovial tissue, synovial fluid, and blood confirmed that
58 PD-1^{hi} CXCR5⁻ cells from all 3 sources contained little, if any, CXCR5 mRNA
59 (**Extended Data Fig. 1d,e**). Thus, seropositive RA synovium contains abundant
60 PD-1^{hi} CD4⁺ T cells that are not Tfh cells.

61

62 Intriguingly, PD-1^{hi} CXCR5⁻ CD4⁺ T cells with a similar multidimensional
63 phenotype also appeared in the circulation, albeit at much lower frequencies
64 (**Fig. 1c, Extended Data Fig. 2a**). Quantification of circulating PD-1^{hi} CXCR5⁻
65 memory CD4⁺ T cells in patients with established seropositive RA (n=42),
66 seronegative RA (n=16), spondyloarthropathies (n=11), and non-inflammatory

67 controls (n=35) demonstrated a significantly increased frequency of PD-1^{hi}
68 CXCR5⁻ cells specifically in seropositive RA patients (**Fig. 1h, Extended Data**
69 **Fig. 2b**, patient characteristics in **Extended Data Table 2**). PD-1^{hi} MHC II⁺
70 CXCR5⁻ and PD-1^{hi} ICOS⁺ CXCR5⁻ cells were also increased in blood of
71 seropositive RA patients (**Extended Data Fig. 2c**). In contrast, the frequencies of
72 PD-1^{hi} CXCR5⁺ cells and cells with intermediate PD-1 expression were not
73 increased (**Extended Data Fig. 2d,e**).

74
75 PD-1^{hi} CXCR5⁻ cell frequencies were more robustly increased in seropositive RA
76 patients with moderate or high disease activity (clinical disease activity index
77 (CDAI)>10), compared to patients with low disease activity (CDAI≤10) (**Fig. 1i**).
78 The frequency of PD-1^{hi} CXCR5⁻ cells did not vary with other clinical parameters
79 such as age, sex, disease duration, use of methotrexate or biologic therapies, or
80 serum anti-CCP antibody titer (**Extended Data Fig. 2f-h**). In an independent
81 cohort of 23 seropositive RA patients assayed before and after starting a new RA
82 medication, there was a significant correlation between reduction in disease
83 activity and reduction in the frequency of PD-1^{hi} CXCR5⁻ T cells (**Extended Data**
84 **Fig. 2i**). The frequency of PD-1^{hi} CXCR5⁻ cells, PD-1^{hi} MHC II⁺ CXCR5⁻ and PD-
85 1^{hi} ICOS⁺ CXCR5⁻ cells decreased significantly in the 18 patients whose disease
86 activity improved after treatment escalation (**Fig. 1j, Extended Data Fig. 2j**).

87
88 Since high PD-1 expression is often considered indicative of an exhausted
89 state^{8,9}, we assessed the function of synovial PD-1^{hi} CXCR5⁻ cells. Surprisingly,

90 despite lack of CXCR5, PD-1^{hi} CD4⁺ T cells sorted from seropositive RA synovial
91 fluid showed >100-fold increased mRNA expression of IL-21 and >1000-fold
92 increased expression of CXCL13, as well as higher levels of IFN- γ and IL-10,
93 compared to PD-1⁻ T cells, with the highest expression in PD-1^{hi} MHC II⁺ cells
94 (**Fig. 2a**, sorted as in **Extended Data Fig. 1d**). In contrast, IL-2 showed a trend
95 towards lower expression in PD-1^{hi} cells.

96

97 Consistent with mRNA expression, PD-1^{hi} CXCR5⁻ cells sorted from RA synovial
98 fluid more frequently produced IL-21 (~30%), but less frequently produced IL-2,
99 compared to PD-1⁻ or PD-1^{int} cells, after stimulation with PMA+ionomycin (**Fig.**
100 **2b**). Optimal CXCL13 production was detected after 24 hours of stimulation with
101 anti-CD3/CD28 beads. Strikingly, at this timepoint, ~25% of PD-1^{hi} CXCR5⁻ cells
102 produced CXCL13, but not IL-2, compared to <1% of PD-1⁻ or PD-1^{int} cells. High
103 IL-21 and CXCL13 production by synovial fluid PD-1^{hi} CXCR5⁻ cells indicates that
104 these cells are not globally exhausted, and instead suggested possible B cell-
105 helper function.

106

107 In support of possible B cell helper-function, PD-1^{hi} MHC II⁺ cells in seropositive
108 RA synovial fluid also expressed high mRNA levels of the transcription factors
109 MAF and BATF and the signaling adaptor SAP (encoded by SH2D1A). These 3
110 factors are important for Tfh cell development or function (**Fig. 2a**)⁴. However,
111 Bcl6, a transcription factor characteristically expressed in Tfh cells, was not

112 elevated in synovial fluid PD-1^{hi} cells, while Blimp-1, a transcription factor
113 typically downregulated in Tfh cells, was upregulated^{4,10}.
114
115 Intracellular flow cytometry confirmed that Blimp-1 was significantly elevated in
116 PD-1^{hi} CXCR5⁻ cells, but not PD-1^{hi} CXCR5⁺ cells, from seropositive RA synovial
117 samples (**Fig. 2c**). In contrast, Bcl6 was dramatically elevated in PD-1^{hi} CXCR5⁺
118 cells, such that the Bcl6/Blimp-1 ratio was uniquely elevated in synovial PD-1^{hi}
119 CXCR5⁺ cells. Expression of MAF, a factor that promotes IL-21 production in
120 human CD4⁺ T cells¹¹, was elevated in both PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺
121 cells.
122
123 PD-1^{hi} CD4⁺ T cells from peripheral blood showed a transcriptional pattern similar
124 to that in synovial fluid PD-1^{hi} cells, with increased expression of IL-21, CXCL13,
125 IFN- γ , MAF, SAP, and Blimp-1, but not IL-2 or Bcl6, in circulating PD-1^{hi} MHC II⁺
126 cells compared to PD-1⁻ cells (**Extended Data Fig. 3a,b**). Both PD-1^{hi} CXCR5⁻
127 and PD-1^{hi} CXCR5⁺ cells expressed increased IL-21 and CXCL13 and
128 decreased IL-2 compared to PD-1⁻ T cells (**Extended Data Fig. 3b**). However,
129 Blimp-1 expression was ~3-fold higher in blood PD-1^{hi} CXCR5⁻ cells compared to
130 PD-1^{hi} CXCR5⁺ cells, while Bcl6 expression was similar. Consistently, after *in*
131 *vitro* stimulation, blood PD-1^{hi} CXCR5⁻ cells expressed more Blimp-1 and less
132 Bcl6 protein than did PD-1^{hi} CXCR5⁺ populations (**Extended Data Fig. 3c**).
133 Taken together, these results indicate that both synovial and blood PD-1^{hi}

134 CXCR5⁻ cells express factors associated with B cell-helper function without an
135 elevated Bcl6/Blimp-1 expression ratio.

136

137 To compare PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ cells more broadly, we analyzed
138 blood PD-1^{hi} cells by mass cytometry (**Extended Data Table 1**). viSNE
139 visualization of blood CD4⁺ T cells clustered PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺
140 cells in close proximity, indicating a similar multidimensional phenotype (**Fig. 3a**,
141 **Extended Data Fig. 4a**). In contrast, FoxP3⁺ T regulatory cells aggregated in a
142 separate region, indicating that most PD-1^{hi} cells are not T regulatory cells, a
143 finding confirmed by flow cytometry (**Fig. 3a**, **Extended Data Fig. 4b**).

144

145 Both PD-1^{hi} CXCR5⁻ cells and PD-1^{hi} CXCR5⁺ cells showed significantly
146 increased expression of 11 proteins, including TIGIT, ICOS, CD38, and CD57,
147 and significantly decreased expression of 5 proteins, including CD25 and CD127
148 (**Fig. 3b**). Unlike TIGIT, additional inhibitory receptors TIM-3, LAG-3, and CTLA-4
149 did not appear enriched on PD-1^{hi} CXCR5⁻ cells (**Extended Data Fig. 4c**).

150 Compared to PD-1^{hi} CXCR5⁺ cells, PD-1^{hi} CXCR5⁻ cells showed lower
151 expression of CCR7 and CD27 but higher CD44 and T-bet (**Fig. 3b, c**),
152 suggesting a potentially distinct migratory capacity^{12,13}.

153

154 We next performed an unbiased transcriptomic comparison of blood PD-1^{hi}
155 CXCR5⁻ and PD-1^{hi} CXCR5⁺ cell subpopulations by low-input RNA sequencing¹⁴.
156 Principal components analysis (PCA) revealed that PD-1^{hi} populations that co-

157 expressed ICOS and/or MHC II were similarly separated from PD-1⁻ cells along
158 the first principal component (PC1), irrespective of CXCR5 expression (**Fig. 3d**,
159 gated in **Extended Data Fig. 4d**). However, PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺
160 cell populations were largely distinguished by the second principal component
161 (PC2), indicating considerable differences in the global transcriptomes of PD-1^{hi}
162 CXCR5⁻ cells and PD-1^{hi} CXCR5⁺ cells beyond CXCR5 expression alone.

163

164 Sixty-six genes were significantly differentially expressed when comparing all of
165 the PD-1^{hi} populations to the PD-1⁻ populations (log fold change >1.2, FDR<0.01,
166 **Extended Data Table 4**), including MAF, TIGIT, and SLAMF6^{15,16}. Analysis of a
167 curated list of Tfh-associated genes^{15,17,18} demonstrated similar upregulation of
168 multiple genes in the pooled PD-1^{hi} CXCR5⁺ cell samples and PD-1^{hi} CXCR5⁻
169 cell samples (**Fig. 3e**), and hierarchical clustering of all 8 cell populations based
170 on this gene list perfectly segregated all PD-1^{hi} populations from PD-1⁻
171 populations, regardless of CXCR5 expression (p<0.026, **Extended Data Fig.**
172 **4e**). These results highlight a shared transcriptional program associated with B
173 cell-helper function in PD-1^{hi} CXCR5⁻ cells and Tfh cells.

174

175 However, we also identified 16 genes with significantly different expression
176 between PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ cells (**Extended Data Table 5**).
177 Notably, PD-1^{hi} CXCR5⁻ cells showed 34-fold increased expression of CCR2, a
178 chemokine receptor that mediates migration to sites of peripheral inflammation¹⁹.
179 A targeted analysis of chemokine receptor expression on PD-1^{hi} CXCR5⁻ cells

180 demonstrated striking upregulation of a set of 'inflammatory' chemokine
181 receptors on these cells, including CCR2, CX3CR1, and CCR5, which was
182 confirmed by flow cytometry (**Fig. 3f,g**)²⁰. Notably, ~50% of PD-1^{hi} CXCR5⁻ cells
183 in seropositive RA synovial fluid and synovial tissue expressed CCR2 (**Fig. 3h**).
184 These results indicate that PD-1^{hi} CXCR5⁻ cells can be distinguished from PD-1^{hi}
185 CXCR5⁺ cells not only by the lack of CXCR5 but also by high expression of
186 inflammatory chemokine receptors.

187

188 To investigate the interconversion of PD-1^{hi} cells that express distinct chemokine
189 receptors, PD-1^{hi} CXCR5⁻ CCR2⁻, PD-1^{hi} CXCR5⁻ CCR2⁺, and PD-1^{hi} CXCR5⁺
190 CCR2⁻ cell populations sorted from blood were stimulated *in vitro* and re-
191 evaluated at different timepoints (**Extended Data Fig. 5a,b**). At day 2, CXCR5
192 was transiently induced on both naïve and memory CD4⁺ T cell populations
193 (**Extended Data Fig. 5c**), as previously described²¹. Interestingly, PD-1^{hi} CCR2⁺
194 cells showed the most limited induction of CXCR5. By day 7, the majority of PD-
195 1^{hi} cells that started out CXCR5⁻ CCR2⁺ cells remained CCR2⁺, while less than
196 5% of these cells expressed CXCR5 (**Extended Data Fig. 5d**). Conversely, most
197 PD-1^{hi} cells that started out CXCR5⁺ CCR2⁻ remained CXCR5⁺, and less than
198 5% of these cells acquired CCR2. These results demonstrate that even with
199 powerful TCR stimulation, CXCR5 and CCR2 expression remain persistent,
200 distinguishing features on PD-1^{hi} *in vitro*.

201

202 We next tested directly if PD-1^{hi} CXCR5⁻ CD4⁺ T cells can provide B cell help *in*
203 *vitro*. PD-1^{hi} CXCR5⁻ cells sorted from seropositive RA synovial tissue or synovial
204 fluid induced differentiation of co-cultured memory B cells into plasma cells, while
205 CXCR5⁻ cells without high PD-1 expression did not (**Fig. 4a,b**, sorted as in
206 **Extended Data Fig. 1d**). The limited number of CXCR5⁺ T cells in synovial
207 samples precluded comparison with PD-1^{hi} CXCR5⁺ cells. PD-1^{hi} CXCR5⁻ cells
208 from blood also induced memory B cell differentiation into plasma cells, with
209 comparable activity in PD-1^{hi} CXCR5⁻ CCR2⁻, PD-1^{hi} CXCR5⁻ CCR2⁺, and PD-1^{hi}
210 CXCR5⁺ cells (**Fig. 4b,c**). PD-1^{hi} CXCR5⁻ cells from synovial fluid and blood also
211 enhanced IgG production in the co-cultures (**Fig. 4d**). Neutralization of IL-21
212 inhibited plasma cell differentiation induced by both blood PD-1^{hi} CXCR5⁺ cells
213 and PD-1^{hi} CXCR5⁻ cells by ~90% (**Fig. 4e**). Expression of SLAMF5, a factor
214 important for T-B interactions,³ was elevated on both PD-1^{hi} CXCR5⁻ and PD-1^{hi}
215 CXCR5⁺ cells, and antibody blockade of SLAMF5, but not SLAMF6, completely
216 abrogated plasma cell differentiation and IgG production (**Fig. 4f, Extended Data**
217 **Fig. 6a-c**). Consistent with a link *in vivo*, RA treatment escalation reduced the
218 frequency of circulating plasmablasts in parallel with the reduction in PD-1^{hi}
219 CXCR5⁻ T cells (**Fig. 1j**).

220

221 Finally, we evaluated the localization of PD-1^{hi} T cells in RA synovium by
222 immunofluorescence microscopy. CD3⁺ T cells with bright PD-1 staining were
223 readily identified (**Fig. 4g**). CXCR5 appeared on CD20⁺ B cells and on a minority
224 of PD-1^{hi} T cells that were enriched within lymphoid aggregates (**Fig. 4h,i**).

225 However, PD-1^{hi} CXCR5⁻ cells outnumbered PD-1^{hi} CXCR5⁺ cells within
226 lymphoid aggregates and were ~4-fold more abundant than PD-1^{hi} CXCR5⁺ cells
227 in regions outside of lymphoid aggregates (**Fig. 4i**). Within lymphoid aggregates,
228 both PD-1^{hi} CXCR5⁻ cells and PD-1^{hi} CXCR5⁺ cells were found adjacent to B
229 cells (**Fig. 4h,j**). However, in areas outside of lymphoid aggregates, the majority
230 of PD-1^{hi} cells adjacent to B cells were CXCR5⁻ (**Fig. 4j, Extended Data Fig.**
231 **6d**). These results suggest a unique capacity of PD-1^{hi} CXCR5⁻ T cells to interact
232 with B cells both within lymphoid aggregates and more diffusely throughout the
233 inflamed synovium.

234

235 Here we have defined a PD-1^{hi} CXCR5⁻ CD4⁺ Tph cell population markedly
236 expanded in rheumatoid arthritis that combines B cell helper function with a
237 migratory program targeting inflamed tissues. The abundance of Tph cells in RA
238 synovium highlights the importance of tissue-localized T-B cell interactions²². Tph
239 cells may infiltrate chronically inflamed tissues, which would not be expected to
240 readily recruit Tfh cells, providing a potential mechanism for the initiation of
241 ectopic lymphoid structures²³⁻²⁵. Tph cell production of CXCL13 and IL-21 may
242 recruit both Tfh and B cells, promoting local autoantibody production that may not
243 be reflected in serum, and perhaps modulating other B cell functions such as
244 cytokine production^{7,26-28}. Identification of the Tph cell phenotype considerably
245 expands the spectrum of B cell-helper T cells that may be assessed as
246 biomarkers for autoantibody-associated diseases. Further, high expression of

247 PD-1 on Tph cells may offer a potential strategy for therapeutic targeting of tissue

248 T cell-B cell interactions.

249

250

251

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349 **Supplementary Information** is linked to the online version of the paper at
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351

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364 **Author Contributions**

365 D.A.R conceived of the project, performed experiments, analyzed data, and
366 wrote the manuscript. M.F.G, Y.L, N.T., and F.M. performed experiments and
367 analyzed data. K.S. analyzed the RNA sequencing data. C.F. analyzed mass
368 cytometry data. J.L.M. performed the immunofluorescence microscopy. J.A.L.
369 developed reagents and assisted with mass cytometry. K.W., L.A.H., P.A.N.,
370 M.E.W., Y.C.L., J.S.C., D.J.T., E.M.M., S.M.H., L.T.D., V.P.B., L.B.I., S.M.G.,
371 A.B.P., A.F., and C.D.B participated in study design, patient recruitment, sample

372 acquisition, and review of the data. S.R. co-supervised the project, analyzed
373 data, and co-wrote the manuscript. M.B.B. conceived of the project, supervised
374 the work, analyzed data, and co-wrote the manuscript. All authors discussed the
375 results and commented on the manuscript.

376

377 **Author Information**

378 RNA sequencing data is available at the GEO repository, accession number
379 GSE80253. Reprints and permissions information is available at

380 www.nature.com/reprints. The authors have no competing financial interests.

381 Correspondence and requests for materials should be addressed to M.B.B.

382 (mbrenner@research.bwh.harvard.edu) or D.A.R. (darao@partners.org).

383

384

Metal	Synovial Panel		Blood cell Panel	
	Target	Clone	Target	Clone
89Y	CD45	HI30		
103Rh			Live/Dead	Cell-ID
141Pr	CD27	M-T271	CD27	M-T271
142Nd	CD19	HIB19	CD45RA	HI100
143Nd	RANKL	MIH24	CD44	BJ18
144Nd	CD64	10.1	CD39	A1
145Nd	CD16	3G8	CD16	3G8/B73.1
146Nd	CD8 α	RPA T8	CD8 α	RPA T8
147Sm	FAP	Poly	CD45RO	UCHL1
148Nd	CD20	2H7	CD28	CD28.2
149Sm	CD45RO	UCHL1	CD25	M-A251
150Nd	CD38	HIT2		
151Eu	PD-1	EH12.2H7	PD-1	EH12.2H7
152Sm	CD14	M5E2		
153Eu	CD69	FN50	CD69	FN50
154Sm	CXCR5	J252D4	CXCR5	J252D4
155Gd	CD4	RPA T4	CD4	RPA T4
156Gd	Podoplanin	NC-08	CD73	AD2
158Gd	CD3	UCHT1	CD3	UCHT1
159Tb	CD11c	Bu15	CD57	HCD57
160Gd	FcRL4	413D12	ICOS	C398.4A
161Dy	CD138	MI15		
162Dy	CD90	5E 10	CXCR3	G025H7
163Dy	CCR2	K036C2		
164Dy	Cadherin11	23C6	CD161	HP-3G10
165Ho	FoxP3	PCH101	FoxP3	PCH101
166Er	CD34	581		
167Er	CD146	SHM-57	CD38	HIT2
168Er	IgA	9H9H11	CCR6	G034E3
169Tm	TCR $\gamma\delta$	B1	CCR7	G043H7
170Er	ICOS	C398.4A		
171Yb	CD66b	G10F5	CD127	A019D5
172Yb	IgM	MHM-88	CD122	TU27
173Yb	CD144	BV9	TIGIT	MBSA43
174Yb	MHCII	L243	HLA-DR	L243
175Lu	IgD	IA6-2	Tbet	4B10
176Yb	VCAM-1	STA	Perforin	dG9
195Pt	Live/Dead	Cell-ID		

Extended Data table 1. Mass cytometry panels for analysis of synovial and blood cells

Patient	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
Age	57	54	76	46	46	79	62	63	52	43
Sex	F	F	F	F	F	F	M	M	F	F
Disease Duration (yrs)	13	17	4	8	19	0.5	19	8	N/A	N/A
CDAI	14	9	17	15	21	25	5	9	N/A	N/A
CRP (mg/L)	25	8	8	11	17	19	13	66	76	0.8
Methotrexate	No	Yes	No	No	No	No	No	Yes	No	No
Biologic therapy	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes
Other synthetic DMARD	Yes	No	Yes	No	Yes	No	No	No	Yes	No

Samples from patients 1-6 were used for mass and flow cytometry phenotyping.

Samples from patients 7-10 were also included in flow cytometry phenotyping.

	Control	Seropositive RA	Seronegative RA	SpA
Number	35	42	16	11
Age	61 ± 13	58 ± 14	58 ± 13	48 ± 12
Female	22 (63)	33 (78)	11 (69)	5 (45)
Disease Duration (yrs)	N/A	13 ± 9	14 ± 10	10 ± 6
C-reactive protein (mg/L)	ND	9.3 ± 17.4	6.3 ± 8.5	3.9 ± 4.2
CDAI	ND	13.7 ± 8.1	9.8 ± 7.6	ND
Methotrexate	0	19 (45)	8 (50)	2 (18)
Anti-TNF	0	16 (38)	6 (38)	10 (90)
Other biologics	0	10 (24)	5 (31)	0
Other synthetic DMARDs	0	4 (10)	1 (6)	0

Average ± SD are shown. Parentheses indicate proportion of patients. Other biologics include abatacept, rituximab, tocilizumab, tofacitinib.

	Improved	Not Improved
Number	18	5
Age	49 ± 17	57 ± 10
Female	17 (94)	4 (80)
CDAI Before	17.6 ± 9.3	21.7 ± 8.9
CDAI After	6.3 ± 4.2	25.6 ± 10.2
Started methotrexate	7	4
Started anti-TNF	4	0
Started other biologic	7	1

Average ± SD are shown. Parentheses indicate proportion of patients.

Extended Data table 2. Clinical characteristics of evaluated patients.

Target	Clone	Fluorophore
CD27	TB01	FITC
CXCR3	CEW33D	PE
CD4	RPA-T4	PE-Cy7
ICOS	ISA-3	PerCP-Cy5.5
CXCR5	J252D4	BV421
CD45RA	HI100	BV510
HLA-DR	G46-6	BV605
CD49d	9F10	BV711
PD-1	EH12.2H7	APC
CD3	HIT3A	AlexaFluor700
CD29	TS2/16	APC-Cy7
Live/Dead		Propidium iodide

Extended Data table 3. Flow cytometry panel for identifying PD-1^{hi} cells

Gene	logFC PD-1 ⁻ Vs PD-1 ^{hi}	p-value	adjusted p-value
PD-1	-6.394163674	1.03E-17	2.07E-13
TOX	-3.973939225	7.21E-13	7.21E-09
ITM2A	-1.206121015	4.54E-10	3.02E-06
TIGIT	-1.919479399	1.03E-09	5.15E-06
MAF	-1.424142776	4.43E-09	1.77E-05
CA6	3.052456565	6.13E-09	2.04E-05
CST7	-3.162619611	1.47E-08	3.80E-05
SCML1	3.900002703	1.71E-08	3.80E-05
SCO2	-5.202859453	1.67E-08	3.80E-05
CDCA7	-4.502988841	2.56E-08	5.12E-05
RAB37	-1.574133208	6.90E-08	0.000115075
ICA1	-2.953123584	2.32E-07	0.000323551
EZH2	-3.019907996	2.43E-07	0.000323551
GZMK	-2.753052708	2.68E-07	0.0003348
MAP3K9	-2.362184122	4.44E-07	0.00052219
PFN1	-1.46238995	7.05E-07	0.000704904
SLAMF6	-1.241364005	8.88E-07	0.000817585
EPSTI1	-2.040445226	8.99E-07	0.000817585
NEFL	4.046814543	1.14E-06	0.00098964
CHN1	-3.450144917	1.22E-06	0.001005063
UBE2L6	-1.228575119	1.55E-06	0.001144629
FANCI	-2.790697425	1.77E-06	0.001264215
PSMA4	-1.420908253	2.22E-06	0.001482222
TOX2	-3.637770018	2.72E-06	0.001698657
FABP5	-2.439672325	3.07E-06	0.001806205
ANXA2	-1.269580271	3.38E-06	0.001932684
CTLA4	-1.739000105	4.31E-06	0.002330776
PLAG1	3.599551478	4.77E-06	0.002510472
HVCN1	-3.516538014	5.08E-06	0.002605277
FAM210A	-2.782673649	5.37E-06	0.002683965
ALOX5	3.436967858	5.88E-06	0.002799526
RGS1	-1.204580998	6.09E-06	0.002834562
MYL6B	-3.072692168	8.27E-06	0.003651008
CEP128	-3.16366403	8.18E-06	0.003651008
ENC1	-3.605191323	8.40E-06	0.003651008
MIS18BP1	-2.065611325	8.89E-06	0.003784483
F5	-1.463762553	1.00E-05	0.004049502
FN1	2.504688725	1.07E-05	0.004049502
CXCR3	-3.01503276	1.06E-05	0.004049502
ASB13	3.35115795	1.06E-05	0.004049502
HIST2H2BF	3.678067821	9.85E-06	0.004049502

PRR5L	-2.171927479	1.10E-05	0.004078013
KRT72	3.194468773	1.24E-05	0.004427712
BZRAP1	-1.970565679	1.37E-05	0.004807752
DUSP2	-1.459107208	1.55E-05	0.005330045
DHFR	-2.732987087	1.74E-05	0.005621497
FBXO41	-2.405412912	1.94E-05	0.00614422
CCDC86	-3.430212114	1.99E-05	0.00620599
FCRL3	-1.770956334	2.06E-05	0.00627086
AKR1C3	-3.308816301	2.07E-05	0.00627086
SHMT2	-1.534205662	2.17E-05	0.006375986
DDX54	-1.733666484	2.21E-05	0.006411258
UBE2A	-1.316870902	2.88E-05	0.007682071
ANXA9	2.915422557	2.85E-05	0.007682071
TUBB4B	-1.239062048	3.12E-05	0.008096696
TIMELESS	-2.607726722	3.24E-05	0.008255379
CCL5	-3.355363898	3.26E-05	0.008255379
UQCRC1	-1.286406184	3.42E-05	0.008442364
TBC1D4	-1.300326318	3.49E-05	0.008514635
SYT11	-1.382472289	3.88E-05	0.009230883
PMAIP1	-2.193153793	3.84E-05	0.009230883
DIRC2	-2.784076776	4.00E-05	0.009304666
SOX8	1.93871557	4.26E-05	0.009799307
SPG20	1.801986978	4.50E-05	0.009899027
DPP3	-1.957767214	4.39E-05	0.009899027
DUSP4	-2.502643153	4.50E-05	0.009899027

Extended Data table 4. Significantly differentially expressed genes between PD-1⁻ and PD-1^{hi} cells

Gene	logFC PD-1 ^{hi} CXCR5 ⁻ vs PD-1 ^{hi} CXCR5 ⁺	p-value	adjusted p-value
RPL39	-1.234532087	1.42E-07	0.000230324
LSP1	1.200685645	2.65E-07	0.00029438
RPL34	-1.273114838	7.88E-07	0.000477468
TTC4	4.631494115	1.29E-06	0.00063025
LIME1	1.562056436	2.07E-06	0.000920442
CCR2	5.094906534	2.25E-06	0.000957805
ACTN4	2.323677525	2.83E-06	0.001130754
CTSH	2.649046873	3.19E-06	0.001251255
PLAC8	-1.351296215	6.85E-06	0.002360784
GLIPR2	1.396871219	7.14E-06	0.00237986
PRR5	2.819632034	1.02E-05	0.003057343
RGS19	2.456286463	1.19E-05	0.003446459
SAMD3	1.24882546	2.50E-05	0.006505926
FOS	-1.595174448	3.68E-05	0.008767004
ANXA4	2.266594524	4.03E-05	0.009370528
LTK	3.888867594	4.20E-05	0.009656997

Extended Data table 5. Significantly differentially expressed genes between PD-1^{hi} CXCR5⁻ cells and PD-1^{hi} CXCR5⁺ cells

387 **METHODS**

388 **Human subjects research**

389 Human subjects research was performed according to the Institutional Review
390 Boards at Partners HealthCare, Hospital for Special Surgery, or the University of
391 Birmingham Local Ethical Review Committee (Birmingham, UK) via approved
392 protocols with appropriate informed consent as required. Patients with RA fulfilled
393 the ACR 2010 Rheumatoid Arthritis classification criteria. Rheumatoid factor and
394 anti-CCP antibody status, C-reactive protein level, and medication usage were
395 obtained by review of electronic medical records. Biologic therapy was defined as
396 use of anti-TNF, abatacept, rituximab, tocilizumab, or tofacitinib. Synovial tissue
397 samples for mass and flow cytometry were collected from seropositive RA
398 patients undergoing arthroplasty at the Hospital for Special Surgery, New York or
399 at Brigham and Women's Hospital, Boston. Samples with lymphocytic infiltrates
400 on histology were prioritized for analyses. Synovial tissue for microscopy was
401 acquired by synovial biopsy of a clinically inflamed joint from seropositive RA
402 patients within the Birmingham early arthritis cohort (BEACON) at the University
403 of Birmingham, UK.

404

405 Synovial fluid samples were obtained as excess material from a separate cohort
406 of patients undergoing diagnostic or therapeutic arthrocentesis of an
407 inflammatory knee effusion as directed by the treating rheumatologist. These
408 samples were de-identified; therefore, additional clinical information was not

409 available, except for the 3 patients from whom paired synovial fluid and blood
410 were obtained.

411

412 Blood samples for clinical phenotyping were obtained from patients seen at the
413 Brigham and Women's Hospital Arthritis Center. For blood cell analyses in the
414 cross-sectional cohort, CDAI was measured by the treating clinician on the day of
415 sample acquisition. Anti-CCP titers were measured using the Immunoscan
416 CCPLus ELISA (Eurodiagnostica), with a positive result defined as >25 units/mL.
417 For RA patients followed longitudinally, a new treatment was initiated at the
418 discretion of the treating physician, and CDAs were determined at each visit by
419 trained research study staff. Blood samples were acquired before initiation of a
420 new biologic therapy or within 1 week of starting methotrexate. Concurrent
421 prednisone at doses <10mg/day were permitted.

422

423 All synovial fluid and blood samples were subjected to density centrifugation
424 using Ficoll-Hypaque to isolate mononuclear cells, which were cryopreserved for
425 batched analyses. Most phenotypic and transcriptomic analyses of blood T cells
426 were performed on samples from both RA patients and non-inflammatory
427 controls, with similar results unless specifically indicated. *In vitro* PD-1^{hi} T cell
428 interconversion assays and *in vitro* B cell helper-assays using blood T cells were
429 performed using PBMC from blood bank leukoreduction collars from anonymous
430 donors.

431

432 All blood CD4⁺ T cell analyses included only CD45RA⁻ memory CD4⁺ T cells
433 except where naïve (CD45RA⁺) cells are specifically indicated. Here the term
434 'memory' is used to denote an 'antigen-experienced' status indicated by loss of
435 the naïve T cell marker CD45RA. This population includes both resting and
436 activated antigen-experienced T cells. Synovial fluid and tissue analyses also
437 utilize only memory CD4⁺ T cells unless total CD4⁺ T cells are indicated. Naïve T
438 cells constituted <10% of the total population of CD4⁺ T cells in synovial tissue
439 and synovial fluid.

440

441 **Synovial tissue analysis**

442 Synovial samples were acquired from discarded arthroplasty tissue. Synovial
443 tissue was isolated by careful dissection, minced, and digested with 100µg/mL
444 LiberaseTL and 100µg/mL DNaseI (both Roche) in RPMI (Life Technologies) for
445 15 minutes, inverting every 5 minutes. Cells were passed through a 70µm cell
446 strainer, washed, subjected to red blood cell lysis, and cryopreserved in Cryostor
447 CS10 (BioLife Solutions) for batched analyses.

448

449 **Mass cytometry**

450 Cryopreserved disaggregated synovial cells or PBMCs were thawed into RPMI +
451 10% FBS (HyClone). Viability was assessed with rhodium for PBMCs and
452 cisplatin (both Fluidigm) for synovial cells. Cells were then washed and stained
453 with primary antibody cocktails at 1:100 dilution (Extended Data Table 4). All
454 antibodies were obtained from the Longwood Medical Area CyTOF Antibody

455 Resource Core (Boston, MA). Cells were then washed, fixed and permeabilized
456 using the Ebioscience Transcription Factor Fix/Perm Buffer for 45 minutes,
457 washed in PBS/1%BSA/0.3% saponin, then stained for intracellular markers.
458 Cells were re-fixed in formalin (Sigma), washed with Milli-Q water, and analyzed
459 on a CyTOF2 for PBMC or Helios (Fluidigm) for synovial cells. Mass cytometry
460 data were normalized using EQ™ Four Element Calibration Beads (Fluidigm) as
461 described²⁹.

462
463 viSNE analyses were performed on cytometry data from 3 of 6 synovial tissue
464 samples, 3 of 9 synovial fluid samples, and 8 of 14 blood samples using the
465 Barnes-Hut SNE implementation on Cytobank (www.cytobank.org). All 3
466 individual synovial tissue sample analyses are shown. For synovial fluid and
467 blood cell analyses, one representative patient sample is shown. For synovial
468 tissue mass cytometry data, gated CD4⁺ T cells were analyzed using all available
469 protein markers, and each synovial tissue sample was analyzed individually to
470 allow for maximal resolution. For paired synovial fluid-blood flow cytometry data,
471 gated memory CD4⁺ T cells from synovial fluid and blood were analyzed together
472 in a single viSNE analysis for direct comparison using an equal number of
473 randomly selected cells from each sample. For blood mass cytometry analyses,
474 equal numbers of gated memory CD4⁺ T cells from each sample were analyzed
475 together using all markers except those used for gating (CD3, CD4, CD45RO).
476 Comparison of marker expression on PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ cells
477 was performed with R-3.2 using permutation Wilcoxon rank-sum tests adjusted

478 for multiple testing. For graphical representation of differential expression, mass
479 cytometry data were transformed using the inverse hyperbolic sine³⁰.

480

481 **Flow cytometry and cell sorting**

482 For phenotypic analyses, cryopreserved cells were thawed into warm RPMI/10%
483 FBS, washed once in cold PBS, and stained in PBS/1% BSA with antibody mixes
484 as in Extended Data Table 2 for 45 minutes. Additional antibodies used include
485 SLAM-AF488 (A12), SLAMF5-PE (CD84.1.21), SLAMF6-PE (NT-7), CCR2-PE
486 (K036C2), CX3CR1-FITC (2A9-1), CD38-PE (HIT2), CD138-PE/Cy7 (MI15),
487 CTLA4-PerCP/Cy5.5 (L3D10) from BioLegend, CCR5-FITC (2D7) and FoxP3-
488 AF647 (236A/E7) from BD Biosciences, LAG-3-APC from R&D Systems, TIM-3-
489 PE/Cy7 (F38-2E2) and TIGIT-PE (MBSA43) from eBioscience.

490

491 Cells were washed in cold PBS, passed through a 70-micron filter, and data
492 acquired on a BD FACSAria Fusion, BD Fortessa, or BD Canto II analyzer using
493 FACSDiva software. Data were analyzed using FlowJo 10.0.7. For blood cell
494 quantification in Figure 2, samples were analyzed in uniformly processed batches
495 of coded samples with multiple disease conditions included in each batch. Upon
496 data acquisition, disease categories were assigned to data files. A single set of
497 gates for PD-1, CXCR5, ICOS, and MHC II was applied to all samples. The
498 percentage of PD-1^{hi} T cell populations among memory CD4⁺ T cells populations
499 and the percentage of plasmablasts (CD19⁺ CD20^{lo} CD38^{hi} CD27⁺) among total
500 CD19⁺ B cells were calculated for indicated samples.

501

502 T cells were sorted directly from synovial fluid and synovial tissue samples. For
503 sorting blood T cells, total CD4⁺ T cells were first isolated by magnetic bead
504 negative selection (Miltenyi Biotec). Cell sorting was performed on a BD
505 FACSAria Fusion sorter using a 70 micron nozzle. Sort gates were drawn as
506 depicted in **Extended Data Fig. 1d**. Cell purity was routinely >98%. For
507 functional analyses, cells were sorted into cold RPMI/10% FBS. For RNA
508 analyses, sorted cells were lysed in RLT lysis buffer (Qiagen) with 1% β-
509 mercaptoethanol (Sigma).

510

511 **Intracellular cytokine staining**

512 Synovial fluid mononuclear cells were stained with anti-PD-1-PE/Dazzle 594,
513 CXCR5-BV605, and CD4-BV650 (Biolegend), and propidium iodide. CXCR5⁺ PD-
514 1^{hi}, PD-1^{int}, and PD-1⁺ CD4⁺ T cells sorted as above were pelleted by
515 centrifugation and resuspended in RPMI/10% FBS at a density of 5x10⁵ cells/mL
516 in 24-well plates. Cells were stimulated with either anti-CD3/anti-CD28 beads at
517 a ratio of 2:1 (cell:bead) for 24 hours, or with PMA (50ng/mL) and ionomycin
518 (1μg/mL). Brefeldin A and monensin (both 1:1000, eBioscience) were added for
519 the last 5 hours. Cells were washed twice in cold PBS, incubated for 30 minutes
520 with Fixable Viability Dye eFluor 455UV (eBioscience), washed in PBS/1%BSA,
521 and then fixed and permeabilized using the eBioscience Transcription Factor
522 Fix/Perm Buffer. Cells were washed in PBS/1%BSA/0.3% saponin and incubated
523 with IL-21-APC (3A3-N2), IL-2-PE/Cy7 (MQ1-17H12), and CXCL13-AF700

524 (53610, R&D Systems) for 30 minutes, washed once, filtered, and data acquired
525 on a BD Fortessa analyzer.

526

527 **Intracellular transcription factor staining**

528 Synovial tissue and synovial fluid cells were thawed, washed twice in PBS, and
529 incubated with Fixable Viability Dye eFluor 455UV (eBioscience) for 30 minutes.

530 Cells were then washed in PBS/1%BSA and stained with antibodies against
531 surface markers anti-CD3-AF700, anti-CD4-BV650, anti-CCR2-PE, anti-CXCR5-
532 BV421, anti-PD-1-PE/Dazzle 594 (all Biolegend) for 30 minutes. Cells were
533 washed once and incubated with eBioscience Transcription Factor Fix/Perm
534 Buffer. Cells were washed in PBS/1%BSA/0.3% saponin and incubated in
535 intracellular antibodies anti-MAF-PerCP-eFluor710 (sym0F1, eBioscience), anti-
536 Bcl6-APC (BCL-UP, eBioscience), and anti-Blimp-1-AF488 (646702, R&D
537 Systems) at 1:20 dilutions for 4 hours. Cells were washed once, filtered, and data
538 acquired on a BD Fortessa analyzer. Intracellular detection of FoxP3 and CTLA-4
539 were performed by the same method on magnetic-bead purified blood CD4⁺ T
540 cells using the indicated surface markers.

541

542 **RT-PCR analyses**

543 RNA isolated using RNeasy Micro Kits (Qiagen). cDNA was prepared using
544 Quantitect RT-PCR (Qiagen) and PCR performed with Brilliant III SYBRGreen on
545 an a Stratagene Mx3000. Primers used were as follows: RPL13A (Forward: 5'-
546 CATAGGAAGCTGGGAGCAAG-3'; Reverse: 5'-GCCCTCCAATCAGTCTTCTG-3'), IL-2

547 (Forward: 5'-AGAACTCAAACCTCTGGAGGAAG-3'; Reverse: 5'-
548 GCTGTCTCAGCATATTCACAC-3'), IFN- γ (Forward: 5'-GCATCGTTTTGGGTTCTCTTG-3';
549 Reverse: 5'-AGTTCCATTATCCGCTACATCTG-3'), IL-10 (Forward: 5'-
550 CGCATGTGAACTCCCTGG-3'; Reverse: 5'-TAGATGCCTTTCTCTTGGAGC-3'), IL-21
551 (Forward: 5'-AGGAAACCACCTTCCACAAA-3'; Reverse: 5'-
552 GAATCACATGAAGGGCATGTT-3'), CXCL13 (Forward: 5'-TCTCTGCTTCTCATGCTGCT-3';
553 Reverse: 5'-TCAAGCTTGTGTAATAGACCTCCA-3'), PD-1 (Forward: 5'-
554 CCAGGATGGTTCTTAGACTCC-3'; Reverse: 5'-TTTAGCACGAAGCTCTCCGAT-3'), CXCR5
555 (Forward: 5'-GGGAGCCTCTCAACATAAGAC-3'; Reverse: 5'-
556 CCAATCTGTCCAGTTCCCAGA-3'), MAF (Forward: 5'-CCGTCCTCTCCCGAGTTTTT-
557 3'; Reverse: 5'-TGCTGGGGCTTCCAAAATGT-3'), Bcl6 (Forward: 5'-
558 GTTTCCGGCACCTTCAGACT-3'; Reverse: 5'-CTGGCTTTTGTGACGGAAAT-3'), BATF
559 (Forward: 5'-TGGCAAACAGGACTCATCTG-3'; Reverse: 5'-CTGTTTCTCCAGGTCTTCGC-
560 3'), SAP (Forward: 5'-GCTATTTGCTGAGGGACAGC-3'; Reverse: 5'-
561 TGTCTGGGACACTCGGTATG-3'), Blimp-1 (Forward: 5'-AACTTCTTGTGTGGTATTGTCCG-
562 3'; Reverse: 5'-TCTCAGTGCTCGGTTGCTTT-3'). Expression levels relative to control
563 gene RPL13A were calculated.

564

565 **RNA sequencing**

566 RNA was isolated from 800-1000 cells from sorted T cell subpopulations as
567 described. 5 μ L of total RNA were placed in wells of a 96-well plate and RNA
568 sequencing libraries were prepared at Broad Technology Labs at the Broad
569 Institute of Harvard and MIT using the Illumina SmartSeq2 platform. Samples

570 were sequenced on a NextSeq500 using 75bp paired-end reads to an average
571 depth of 9M pairs of reads per sample. All cDNA transcripts from Ensembl
572 release 82 were quantified with Kallisto version 0.42.4³¹. We used limma to
573 model each gene as a linear combination of donor-specific effects. The residuals
574 from these models were tested by ANOVA across 8 gates, and 581 genes with a
575 significant F statistic with <5% FDR were selected for PCA. Heatmaps show row-
576 normalized relative gene expression z-scores across columns (mean 0 and
577 variance 1), with subpopulations of PD-1^{hi} CXCR5⁻ or PD-1^{hi} CXCR5⁺ averaged
578 to yield overall PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ expression values. In
579 comparisons of specific cell populations, genes with log fold change >1.2 and
580 FDR <1% were considered differentially expressed.

581

582 **PD-1^{hi} cell *in vitro* stimulation assays**

583 CD4⁺ T cells were purified from PBMCs from leukoreduction collars by magnetic
584 bead negative selection and stained with anti-CD4-BV650, anti-CD45RA-BV510,
585 anti-PD-1-APC, anti-CXCR5-BV605, and anti-CCR2-PE/Cy7. Naïve CD4⁺ T cells
586 and memory CD4⁺ T cell subpopulations were sorted into RPMI/10% FBS.
587 50,000 cells were resuspended in RPMI/10%FBS at 0.25 x 10⁶ cells/mL and
588 cultured with anti-CD3/CD28 beads (Dynabeads) at a cell:bead ratio of 5:1 for 2
589 or 7 days. Cells were then either re-stained with anti-PD-1-PE and anti-CXCR5-
590 BV421 antibodies and sorted into lysis buffer for RT-PCR analyses, or stained
591 with CCR2-PE and CXCR5-BV421 and analyzed by intracellular flow cytometry
592 for transcription factors as above.

593

594 **T cell-B cell co-cultures**

595 Total B cells were isolated first from PBMCs from blood bank leukoreduction
596 collars by magnetic bead positive selection using CD19 (Miltenyi), then CD4⁺ T
597 cells were isolated by negative selection. B cells were stained with CD14-APC,
598 CD3-PeCy7, and CD27-BV510 (all from Biolegend), and memory B cells sorted
599 as CD27⁺ CD14⁻ CD3⁻ cells on a BD FACSAria Fusion to remove contaminating
600 T cells and monocytes. Sorted T cell populations were co-cultured with
601 autologous memory B cells at a ratio of 1:10 in 100uL of RPMI/10%FBS and
602 stimulated with LPS (5µg/mL) and SEB (1µg/mL) for 7 days. For co-cultures
603 using synovial tissue or synovial fluid T cells, allogeneic memory B cells from
604 PBMC were used. Supernatants were collected and total IgG measured by
605 ELISA (eBioscience). Cells were harvested and analyzed by flow cytometry, with
606 plasmablasts defined as CD19⁺ CD20^{low} CD38^{hi} CD27⁺ and plasma cells defined
607 as CD19⁺ CD20^{low} CD38^{hi} CD27⁺ CD138⁺. For blocking experiments, 10µg/ml
608 anti-SLAMF5 or anti-SLAMF6 antibodies (Biolegend) or 20µg/mL IL-21R-Ig (R&D
609 Systems) were used.

610

611 **Immunofluorescence microscopy**

612 6 micron sections of synovium frozen in OCT were fixed in acetone, rehydrated
613 in PBS, and blocked with 10% normal goat serum prior to application of primary
614 antibodies as follows: PD-1 (EH12.2H7, BioLegend), CD3 (SP7, Abcam), CD20
615 (L26, Dako), CXCR5 (MAB190, R&D Systems), all at a dilution of 1:100 except

616 for CD20, which was used at 1:300. All secondary antibodies were raised in goat.
617 CXCR5 was detected using anti mouse IgG2b biotin (Southern biotech) followed
618 by streptavidin conjugated AlexaFluor 546 (Life Technologies), CD20 with anti-
619 mouse IgG2a FITC (both Southern Biotech), PD-1 with anti-mouse IgG1
620 conjugated to AlexaFluor 647 and CD3 with anti-rabbit AlexaFluor 546 (both Life
621 Technologies). FITC staining was amplified with anti-FITC AlexaFluor 488 (Life
622 Technologies). Slides were mounted using ProLong Diamond (Life
623 Technologies), left to cure overnight and imaged using a Zeiss LSM 780 confocal
624 microscope. Images were processed using Zen Black (Zeiss) and then ImageJ.
625 Cell counts were performed on images obtained from confocal imaging using the
626 Cell Counter plugin for ImageJ (imagej.net/Cell_Counter). Synovial regions were
627 categorized as 'lymphoid aggregates' when the B cells and T cells formed distinct
628 clusters, and 'diffusely infiltrated' when B cells were loosely distributed within the
629 synovium.

630

631 **Statistical analyses**

632

633 Statistical tests were performed as indicated in figure legends using two-sided
634 tests. Dunn's test was used for multiple comparisons in non-parametric tests and
635 Bonferroni test for ANOVA. P-values <0.05 were considered significant.

636

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FIGURE LEGENDS:

Figure 1: Expanded PD-1^{hi} CXCR5⁻ CD4⁺ T cells in joints and blood of seropositive RA patients.

a) viSNE plots of RA synovial tissue total CD4⁺ T cells analyzed by mass cytometry. Color indicates cell expression level of labeled marker. Dotted circle indicates PD-1^{hi} cells. Arrow indicates CXCR5⁺ cells. b) PD-1^{hi} T cell frequency in RA synovial tissue by mass cytometry (n=6). c) viSNE plots of paired RA synovial fluid and blood memory CD4⁺ T cells. d) Flow cytometric detection of synovial fluid PD-1^{hi} CD4⁺ T cells. e) PD-1^{hi} CD4⁺ T cell frequency in synovial fluid from seropositive RA (n=9) and seronegative inflammatory arthritides (n=19). f) Flow cytometry for PD-1 and CXCR5 on memory CD4⁺ T cells. g) Frequency of PD-1^{hi} cells in seropositive RA synovial fluid (n=9) and tissue (n=10). h) Frequency of PD-1^{hi} CXCR5⁻ cells in seropositive RA (n=42), seronegative RA (n=16), spondyloarthropathies (SpA, n=11), and control (n=35) patient blood. i) PD-1^{hi} frequency in seropositive RA patients with low (n=14) or moderate-high (n=28) disease activity. j) PD-1^{hi} CD4⁺ T cell and plasmablast frequencies before and after RA treatment escalation (n=18). Mean \pm SD in b,e,g, median \pm interquartile range in h,i shown. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 by Mann-Whitney (e,g), Kruskal-Wallis (h,i), Wilcoxon test (j).

Figure 2: Synovial PD-1^{hi} CXCR5⁻ CD4⁺ T cells express factors associated with B cell help.

a) RT-PCR for cytokines (n=7 donors) and intracellular regulators (n=5 or 6 donors) in T cell populations from seropositive RA synovial fluid. Median \pm interquartile range. b) Flow cytometric quantification of IL-21, IL-2, and CXCL13 production by stimulated synovial CD4⁺ T cell (n=3 experiments using different donors). c) Flow cytometric quantification of transcription factor expression in CD4⁺ T cells from RA synovial fluid (blue, n=3 donors) or synovial tissue (green, n=3 donors). For b,c, mean \pm SD shown. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 by Friedman's test compared to PD-1⁻ MHC II⁻ cells (a) or one-way ANOVA comparing PD-1⁻ CXCR5⁻, PD-1^{hi} CXCR5⁻, and PD-1⁻ CXCR5⁺ (c).

Figure 3: High dimensional analyses of PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ cells identify shared and distinct features.

a) viSNE plots of blood memory CD4⁺ T cells from a representative RA patient. Circle indicates PD-1^{hi} cells. b) Transformed median expression difference of significantly altered proteins between PD-1^{hi} populations and PD-1⁻ CXCR5⁻ memory CD4⁺ T cells (n=14 RA patients). c) Median expression of indicated proteins (n=7 RA patients (black) and 7 controls (grey)). d) PCA of RNA-seq transcriptomes (n=4 donors). e,f) Heatmap of expression of Tfh-associated genes (e) or chemokine receptors (f) by RNA-seq. g) Flow cytometric quantification of chemokine receptor expression on blood memory CD4⁺ T cells. h) CCR2 expression on PD-1^{hi} CXCR5⁻ CD4⁺ T cells in RA synovial samples (tissue n=10, fluid n=5). For c,g,h, mean \pm SD shown. * p<0.01, ** p<0.001, *** p<0.0001 Wilcoxon test.

Figure 4: PD-1^{hi} CXCR5⁻ cells promote plasma cell differentiation via IL-21 and SLAMF5 interactions.

a) Flow cytometric detection of plasma cells. b) Plasma cell frequency in co-cultures of memory B cells with indicated T cell populations from indicated sources.

Pooled data from 2 experiments (synovial tissue, n=3 replicates per experiment), 3 experiments (synovial fluid), or 6 experiments (blood) using different donors. c) Co-cultures using blood T cell subpopulations as in (b). d) Total IgG in supernatants of co-cultures as in (b). e,f) Co-cultures as in (b) with IL-21R-Ig fusion protein (e) or anti-SLAMF5/SLAMF6 antibody (f). For c-f) 1 of 3 experiments with different donors (n=3 replicates) shown. g,h)

Immunofluorescence microscopy of RA synovium showing PD-1^{hi} CXCR5⁻ cells (white arrow) and PD-1^{hi} CXCR5⁺ cell (gray arrow). Scale bar = 50 microns. i,j) Quantification of PD-1^{hi} cells (i) and PD-1^{hi} cells adjacent to B cells (j) in RA synovium (n=5-8 HPF from 4 samples). Means \pm SD shown. * p<0.05, ** p<0.01, *** p<0.001 Mann-Whitney (b, synovial tissue), Kruskal-Wallis compared to PD-1⁻ CXCR5⁻ (b,c,e,f), or Wilcoxon (g).

Extended Data Figure 1: Detection of PD-1^{hi} CD4⁺ T cells in RA synovial tissue and fluid by mass and flow cytometry.

a) viSNE plots of mass cytometry data on CD4⁺ T cells as in Fig. 1a from two additional seropositive RA synovial tissue samples. b) Gating strategy to identify

synovial tissue PD-1^{hi} CD4⁺ T cell populations by mass cytometry. c) Gating strategy to identify synovial fluid PD-1^{hi} memory CD4⁺ T cells by flow cytometry. d) Examples of gating used to sort memory CD4⁺ T cell populations from patient samples. e) Detection of CXCR5 mRNA by RT-PCR in sorted memory CD4⁺ T cell populations from synovial tissue (n=3 donors, 2 of which provided sufficient PD-1^{hi} CXCR5⁺ cells for analysis), synovial fluid (n=3 donors, 1 of which provided sufficient PD-1^{hi} CXCR5⁺ cells for analysis), and blood (n=2 donors). Purple boxes indicate PD-1⁻ and PD-1^{hi} CXCR5⁺ cells sorted from human tonsil as controls. Lines in (e) indicate mean for synovial or blood samples.

Extended Data Figure 2: PD-1^{hi} CXCR5⁻ CD4⁺ T cells are expanded in circulation of patients with active, seropositive RA and decrease with response to therapy.

a) Mean expression of MHC II and ICOS in memory CD4⁺ T cell populations defined by PD-1 and CXCR5 expression from synovial tissue (n=10), synovial fluid (n=9), and blood (n=42) from seropositive RA patients. Mean ± SD shown. b) Flow cytometric detection of PD-1 and CXCR5 expression on blood memory CD4⁺ T cells. c) Frequency of PD-1^{hi} CXCR5⁺ cells within circulating memory CD4⁺ T cells in patients with seropositive RA (RA Ab⁺, n=42), seronegative RA (RA Ab⁻, n=16), spondyloarthropathies (SpA, n=11), and non-inflammatory control patients (control, n=35) as in Fig. 1h. d,e) Frequency of PD-1^{hi} subpopulations that co-express MHC II or ICOS (d) or with intermediate PD-1 expression (e) in patients as in (c). f) Correlation between age or disease

duration and circulating PD-1^{hi} CXCR5⁻ CD4⁺ T cells in all seropositive patients for which data was available (n=38). g) PD-1^{hi} CXCR5⁻ T cell frequencies in seropositive RA patients segregated based on sex or medication usage (n=38). h) Correlation between serum anti-CCP antibody titer and circulating PD-1^{hi} CXCR5⁻ CD4⁺ T cell in all RA patients (n=53, black line, p=0.0049) or in only anti-CCP antibody⁺ patients (n=29, green line, p=0.48). i) Correlation between fold change in CDAI and fold change in PD-1^{hi} CXCR5⁻ T cell frequency patients 3 months after addition of a new RA medication (n=23; methotrexate=11, anti-TNF=4, abatacept=4, tocilizumab=2, tofacitinib=2). j) Frequency of PD-1^{hi} T cell subpopulations before and after RA treatment escalation in 18 patients with reduced disease activity after therapy. Median ± interquartile range in c,d,e, and mean ± SD in a,g is shown. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 by Kruskal-Wallis (c,e), Mann-Whitney (d,g), Wilcoxon test (j). In f,h,i p-values calculated by Spearman correlation.

Extended Data Figure 3: Blood PD-1^{hi} CXCR5⁻ CD4⁺ T cells express factors associated with B cell help.

a) mRNA expression levels of cytokines/chemokines (n=10 donors, 6 RA patients (black), 4 controls (grey)) or transcription factors/signaling molecules (n=4 or 5 donors) detected by RT-PCR in sorted circulating memory CD4⁺ T cell populations, normalized to RPL13A. Median ± interquartile range shown. * p<0.05, ** p<0.01, *** p<0.001 Friedman's test, compared to PD-1⁻ MHCII⁻ group.

b) Cytokine and transcription factor mRNA expression in blood PD-1^{hi} CD4⁺ T

cell populations divided according to CXCR5 expression, relative to PD-1⁻ memory CD4⁺ T cells (n=6 donors). Mean ± SD shown. c) Flow cytometric quantification of Bcl6 and Blimp-1 in PD-1^{hi} memory CD4⁺ T cell subpopulations sorted according to chemokine receptor expression, then stimulated *in vitro* for 2 days with anti-CD3/CD28 beads. Representative data from 1 of 3 experiments using cells from different donors.

Extended Data Figure 4: Identification and characterization of circulating PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ in mass cytometry and RNA-seq analyses.

a) Gating of blood PD-1^{hi} memory CD4⁺ T cells in mass cytometry analyses. b) Flow cytometric detection of FoxP3 and PD-1 in blood memory CD4⁺ T cells from RA patients (black circles, n=5) and controls (grey circles, n=3). * p<0.05, **p<0.001 Kruskal-Wallis test compared to PD-1⁻ cells. c) Flow cytometric detection of inhibitory receptors on blood memory CXCR5⁻ CD4⁺ T cells. Data from 1 of 3 RA patients with similar results. d) Sort strategy for PD-1^{hi} CXCR5⁻ and PD-1^{hi} CXCR5⁺ cell populations for RNA-seq. e) Hierarchical clustering of T cell subsets sorted as in (d), with clustering based on expression of Tfh-associated genes measured in RNAseq transcriptomes.

Extended Data Figure 5: Limited interconversion of PD-1^{hi} CCR2⁺ and PD-1^{hi} CXCR5⁺ T cells *in vitro*.

a) Flow cytometry of CXCR5 and CCR2 on gated PD-1^{hi} CD4⁺ cells from blood.
b) Expression of CXCR5 and CCR2 on indicated sorted PD-1^{hi} T cell populations 7 days after *in vitro* stimulation with anti-CD3/CD28 beads. c,d) Percentage of cells from each sorted PD-1^{hi} population that expressed CXCR5 or CCR2 on day 2 (c) or day 7 (d) after *in vitro* stimulation. Naive CD4⁺ T cells are shown as control. Mean ± SD shown (n=3 donors from 3 separate experiments).

Extended Data Figure 6: SLAMF5 is required for B cell-helper function of PD-1^{hi} CXCR5⁻ CD4⁺ T cells.

a) Flow cytometric quantification of SLAM, SLAMF5, and SLAMF6 expression on memory CD4⁺ T cells (n=10 donors, 5 RA patients, 5 controls). b) Quantification of frequency of memory B cells with plasma cell markers after co-culture with PD-1^{hi} CXCR5⁺ CD4⁺ T cells with addition of blocking antibodies against SLAMF5 and/or SLAMF6. c) IgG quantification by ELISA in co-cultures of memory B cells with PD-1^{hi} CXCR5⁻ or PD-1^{hi} CXCR5⁺ CD4⁺ T cells with addition of blocking antibodies against SLAMF5 and/or SLAMF6. For b,c) 1 of 3 experiments with similar results (n=3 replicates shown). Mean ± SD shown. * p<0.05, ** p<0.01, *** p<0.001 Kruskal-Wallis compared to PD-1⁻ CXCR5⁻ (a) or isotype control (b,c). d) Immunofluorescence microscopy of CD20 (green), CXCR5 (red), and PD-1 (blue), in seropositive RA synovial tissue. Arrows point to PD-1^{hi} CXCR5⁻ cells adjacent to B cells. Scale bar = 50 microns.