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1 **Increased frequency of CD4+ and CD8+ follicular helper T cells in human lymph node biopsies**
2 **during the earliest stages of rheumatoid arthritis**

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18 **Keywords:** Rheumatoid arthritis, lymph nodes, T-lymphocytes, B-lymphocytes, follicular T helper
19 cells

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22 **ABSTRACT**

23 **Objectives**: Follicular helper T cells (Tfh cells) provide key B cell help, and are essential in germinal
24 center (GC) formation and (auto) antibody generation. To gain more insight into their role during the
25 earliest phase of rheumatoid arthritis (RA) we analyzed their frequencies, phenotype and cytokine
26 profile in peripheral blood and lymphoid tissues.

27 **Methods**: Using flow cytometry, we studied the frequency of Tfh and B cells in peripheral blood and
28 lymph node (LN) needle biopsies. Three donor groups were included and compared: healthy controls
29 (HCs), autoantibody positive individuals at risk for developing RA (RA-risk individuals), and early
30 RA patients. *Ex vivo* stimulation of lymphocytes with PMA/ionomycin was performed to assess
31 cytokine secretion by Tfh cells.

32 **Results**: In blood, the frequency of circular Tfh cells (cTfh) did not differ between study groups. In
33 lymphoid tissue, the frequency of Tfh cells correlated strongly with the frequency of $CD19^+$ B cells.
34 Compared to healthy controls, LN samples of RA patients and RA-risk individuals showed more
35 $CD19^+$ B cells and more $CD4^+CXCR5^+$ and $CD8^+CXCR5^+$ Tfh cells. These Tfh cells from LNs
36 expressed less IL-21 upon *ex vivo* stimulation.

37 **Conclusion**: LN tissue of early RA patients as well as part of RA-risk individuals exhibit increased
38 frequencies of Tfh cells correlating with increased numbers of B cells. Interestingly, IL-21 production
39 is already aberrant in the very early at risk phase of the disease. This suggests that Tfh cells may present
40 a novel rationale for therapeutic targeting during the preclinical stage of the disease to prevent further
41 disease progression.

42

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43 **INTRODUCTION**

44 The presence of autoantibodies years before the presence of clinical signs and symptoms in rheumatoid
45 arthritis (RA) suggest an increase in B cell differentiation towards antibody-producing plasma cells
46 already very early in the disease (1). Such proliferation and differentiation of B cells is supported by
47 $CD4^+$ follicular T helper cells (Tfh cells) (2). These Tfh cells derive from naïve $CD4^+$ T cells.
48 Following TCR stimulation under the influence of receptor-ligand interactions and cytokine milieu, T
49 cells may upregulate CXCR5, causing them to migrate to the CXCL13 expressing follicular border
50 where they interact with B-cells and receive signals that will potentially drive them into Tfh cells (3).
51 Producing cytokines like IL-21 and IL-4, Tfh cells interact with B cells which results in B-cell
52 differentiation towards short-lived plasmablasts or in B-cell migration into the follicles to contribute
53 to the formation of germinal centers (GC) (4). Migration of Tfh cells into the B-cell follicles further
54 supports GC formation and drives B-cell differentiation into memory B-cells or long-lived antibody-
55 secreting plasma cells. Overall, the net effect of this process depends on the balance between
56 inflammatory and regulatory signals and is tightly regulated to prevent aberrant (auto)immune
57 activation (5).

58 One of the studies showing the contribution of $CD4^+$ Tfh cells in autoimmunity used the sanroque
59 mouse model. Sanroque mice lack a repressor of ICOS, Roquin, resulting in excessive Tfh cell
60 formation and subsequent GC formation. These mice have increased titres of autoantibodies and lupus-
61 like symptoms (6). In human studies, SLE patients also show increased frequencies of blood Tfh cells
62 compared with healthy controls (HCs) which correlate with an increase in circulating autoantibodies
63 (7-10). Similarly, increased frequencies of peripheral blood Tfh cells have been detected in type I
64 diabetes and RA patients (11-15). Since Tfh cells can leave the lymphoid tissues and relocate through
65 peripheral blood they may contribute to B-cell differentiation and formation of tertiary lymphoid
66 structures at sites of inflammation.

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67 While the role of $CD4^+$ Tfh cells in autoimmunity is widely accepted, current data regarding a potential
68 role for $CD8^+CXCR5^+$ follicular-like T cells are less clear, and actually point to a dual role. Like $CD4^+$
69 Tfh cells, $CD8^+$ Tfh cells express CXCR5, BCL6, IL-21, ICOS as well as PD-1(16-18). An ex vivo
70 study reported the ability of $CD8^+$ $CXCR5^+$ T cells to induce the apoptosis of $CD4^+$ Tfh cells, resulting
71 in inhibition of IL-21 production (19). However, an antibody-enhancing function of these cells has also
72 been reported in virus-infected mice where IL-21 producing $CXCR5^+ICOSL^+CD8^+$ T cells were shown
73 to enhance the production and class-switching of IgG antibodies, revealing a major role of $CD8^+$ Tfh
74 cells in the immune response (20).

75 Studies into the role of T cells in the pathogenesis of RA in humans have mainly focused on the well-
76 known Th1, Th2, Th17, and Treg subsets (21-23), during established disease, and in cell populations
77 from peripheral blood and inflamed joints (22, 24). Studies of Tfh cells focused on peripheral blood
78 samples or inflamed tissue (4, 25-27), while studies investigating Tfh cells in lymphoid organs during
79 the earliest phases of autoimmunity are lacking. To the best of our knowledge, no study has analysed
80 $CD8^+$ Tfh during the various stages of RA. To gain more insight into the initial activation of Tfh cells
81 in lymphoid tissue and in the role of lymph node Tfh cells during the earliest phases of RA, more
82 research is needed.

83 In this study, we hypothesized that Tfh cells contribute to autoantibody production in the earliest
84 preclinical phases of RA by driving B cell differentiation in secondary lymphoid organs. Using samples
85 acquired by core-needle biopsies of inguinal LNs (28, 29), we analysed and compared the frequencies
86 of $CD4^+$ and $CD8^+$ Tfh cells in the blood and lymphoid tissue from healthy controls (HCs),
87 autoantibody-positive individuals at risk for developing RA (RA-risk individuals) and early RA
88 patients (30).

89

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90 **MATERIALS AND METHODS**

91 *Study subjects*

92 Twenty-four individuals at risk for developing RA (RA-risk) were selected. RA-risk status was defined
93 by the presence of IgM rheumatoid factor (IgM-RF) and/or anti-citrullinated protein antibodies
94 (ACPA) positive) in subjects without any evidence of arthritis (30). The median follow-up time was
95 26.8 months (14.3-39.2 (interquartile range, IQR)) and none of these RA-risk individuals had
96 developed arthritis as yet. We also included 16 early RA patients, based on American College of
97 Rheumatology and European League Against Rheumatism (ACR/EULAR) 2010 criteria (31), who
98 were naïve for disease-modifying antirheumatic drugs (DMARD) and biologicals with a disease
99 duration (defined by having arthritis in any joint) less than 1 year. For comparison, 17 seronegative
100 healthy controls (HCs) were included in the study. The study was performed according to the principles
101 of the Declaration of Helsinki (32), approved by the institutional review board of the Academic
102 Medical Centre of the University of Amsterdam, and all study subjects gave written informed consent.
103 Demographics of all study subjects are listed in table 1.

104

105 *Sample processing and cell culture*

106 Ultrasound-guided inguinal LN biopsies were taken and immediately processed for flow cytometry
107 analysis, snap-frozen en bloc in Tissue-Tek OCT compound (Miles, Elkhart, IN) for
108 immunohistochemistry analysis or snap frozen for RNA isolation as described previously (28). For
109 flow cytometry analyses, LN biopsies were put through a 70 µm cell strainer (BD Falcon, San Jose,
110 CA) to obtain a single cell suspension. Peripheral blood mononuclear cells (PBMC) were isolated using
111 standard density gradient centrifugation using lymphoprep (Nycomed AS, Oslo, Norway) and stored

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112 in liquid nitrogen until further use. Freshly isolated LN cells and thawed PBMC were incubated in
113 RPMI culture medium (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA) for 4 hours
114 in the presence or absence of Phorbol Myristate Acetate (PMA) and Ionomycine with Brefeldin A (all
115 from Sigma Aldrich, St Louis, MO) and Golgi stop (BD Biosciences, San Jose, CA). After 4 hours,
116 cells were washed and analyzed by flow cytometry.

117

118 *Antibodies and flow cytometry analysis*

119 Cells were stained for 30 minutes at 4^0C in PBS containing 0.01% NaN_3 and 0.5% BSA (Sigma
120 Aldrich) with directly labelled antibodies against: CXCR5 alexa fluor 488 (clone RF8B2), CCR7 PE-
121 Cy7 (clone 3D12), CD4 APC-H7 (clone SK3), CD8 V450 (RPA-T8), CD3 V500 (UCHT1) (all from
122 BD Biosciences, San Jose, CA), PD-1 PE (J105), CD45 RA (L307.4) efluor450 (all from eBioscience
123 Inc., San Diego, CA). For intracellular cytokine staining we used IL-10 Pe-Cy7 (clone JES3-9D7)
124 (Biolegend, San Diego, CA) and IL-21 alexa fluor 647 (clone 3A3-N2.1) (BD Biosciences). Cells were
125 analyzed on a FACS Canto II (BD Biosciences) and data were analyzed using FlowJo software
126 (FlowJo, Ashland, OR). Data were plotted as frequency of positive cells.

127

128 *Statistical analysis*

129 After testing for normality with D'Agostino and Pearson omnibus test, data are presented as mean with
130 standard deviation or median with IQR. Differences between groups were analysed using the Kruskall
131 Wallis test or one-way analysis of variances (ANOVA). Correlations were calculated with Spearman's
132 Rank Correlation Coefficient. All statistical analyses were performed using GraphPad Prism Software

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133 (version 6, GraphPad Software, Inc. La Jolla, CA). P-values <0.05 were considered statistically
134 significant.

135

136 **RESULTS**

137 ***The frequency of peripheral blood CD4$^+$ and CD8$^+$ circulating follicular helper T cells is comparable***
138 ***between healthy controls, RA-risk individuals and early RA patients***

139 To analyse the frequency of peripheral blood CD4$^+$ and CD8$^+$ cTfh cells in HCs, RA-risk individuals
140 and early RA patients we first analysed the total number of CXCR5$^+$ and PD-1$^+$ cells within the CD4$^+$
141 and CD8$^+$ T cells (see gating Figure 1A and supplementary figure 1 (negative control)). The
142 frequencies of CD4$^+$CXCR5$^+$ and CD4$^+$PD-1$^+$ T cells as well as CD8$^+$CXCR5$^+$ and CD8$^+$PD-1$^+$ T cells
143 were on average comparable between the three study groups (Figures 1B and C). Within the
144 CD4$^+$CXCR5$^+$ T cell subset, the CCR7^{low}PD1^{high} cells have been described as active Tfh cells and the
145 CCR7^{high}PD1^{low} as quiescent Tfh cells (14). In our analysis the frequency of active (CCR7^{low}PD-1^{high}
146) and quiescent (CCR7^{high}PD-1^{lo}) cTfh cells within the blood CD4$^+$CXCR5$^+$ and CD8$^+$CXCR5$^+$ cells
147 was comparable between the three study groups (Figures 1D and E). No significant correlations
148 between the frequencies of various cTfh cells were present with age or autoantibodies detected in blood
149 (data not shown).

150 Next we analyzed the capacity for cytokine production in blood cTfh cells upon ex vivo stimulation
151 with PMA/ionomycin. The frequency of CD4$^+$CXCR5$^+$IL-21$^+$, CD4$^+$CXCR5$^+$Il-10$^+$,
152 CD8$^+$CXCR5$^+$IL-21$^+$ and CD8$^+$CXCR5$^+$IL-10$^+$ T cells was on average comparable between the three
153 study groups (Figures 1F and G). As expected, the frequency of peripheral blood active-Tfh cells
154 producing Il-21 is higher than the frequency of quiescent-Tfh cells. Finally, the frequency of IL-21$^+$

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155 cells among active $CD4^{+}CXCR5^{+}CCR7^{low}PD\text{-}1^{high}$ and $CD8^{+}CXCR5^{+}CCR7^{low}PD\text{-}1^{high}$ was on
156 average comparable between the three study groups (Figure 1H).

157 Taken together, the frequency of blood $CD4^{+}$ and $CD8^{+}$ cTfh cells is highly variable but on average
158 not different between RA-risk individuals and early RA patients compared with healthy controls (HCs).

159

160 ***$CD4^{+}$ and $CD8^{+}$ follicular helper T cells are increased in lymphoid tissue of RA patients***

161 Next, we analysed the frequencies of $CD4^{+}$ and $CD8^{+}$ Tfh cells based on CXCR5 expression in LN
162 biopsies (see gating Figure 2A). CXCR5 expression defines LN T cells that are capable of moving
163 towards the follicular border where they can interact with B cells (2). Among $CD3^{+}$ T cells, the
164 frequency of total $CD4^{+}$ T cells was not significantly higher in RA-risk individuals compared to HCs,
165 but significantly increased in early RA individuals compared to HCs (Figure 2B). Among $CD4^{+}$ T cells
166 the frequency of $CXCR5^{+}$ Tfh cells is increased in early RA patients compared with HCs (Figure 2B;
167 $p<0.05$), while frequencies in RA at-risk individuals are in between. We next evaluated the frequencies
168 of $CD8^{+}$ T cells. Among $CD3^{+}$ cells, the frequency of $CD8^{+}$ T cells was similar between RA-risk
169 individuals and HCs, while it was significantly lower in early RA patients compared to HCs (Figure
170 2C). Among $CD8^{+}$ T cells, the frequency of $CD8^{+}CXCR5^{+}$ Tfh cells was markedly increased ($p<0.05$)
171 in RA-risk individuals as well as early RA patients when compared to HCs (Figure 2C).

172 Since IL-21 and IL-10 production was below detection limit in unstimulated cells, we analysed IL-21
173 and IL-10 production in LN $CD4^{+}CXCR5^{+}$ and $CD8^{+}CXCR5^{+}$ Tfh cells upon ex vivo stimulation with
174 PMA/ionomycin. Among $CD4^{+}CXCR5^{+}$ Tfh cells, the frequency of IL-21 producing cells upon ex
175 vivo stimulation was significantly decreased in early RA patients ($p<0.01$) when compared with HCs,
176 while the frequency was intermediate for the RA-risk individuals (Figure 2D). Similar findings were

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177 observed for $CD8^+CXCR5^+$ Tfh cells, but without reaching statistical significance (Figure 2D). In
178 contrast, the frequency of IL-10 producers in the $CD4^+$ and $CD8^+$ $CXCR5^+$ Tfh cells was comparable
179 between the three study groups (Figure 2E).

180

181 ***In lymphoid tissue, the frequencies of $CD4^+$ and $CD8^+$ follicular helper T cells correlate with the***
182 ***frequencies of $CD19^+$ B cells***

183 Since $CD4^+CXCR5^+$ and $CD8^+CXCR5^+$ Tfh cells were increased in LNs of RA patients compared to
184 HCs, we investigated if they were in any way related to the frequencies of B cells found in LNs.
185 Consistent with previous findings (29), the frequency of $CD19^+$ B cells was increased in early RA
186 patients compared with healthy controls ($p<0.05$) but similar between healthy controls and RA-risk
187 individuals (Figure 3A). When compared with $CD4^+CXCR5^+$ Tfh cells, we found a strong and
188 significant correlation between the frequencies of $CD19^+$ B cells and $CD4^+CXCR5^+$ Tfh cells
189 ($p<0.0001$, $r=0.76$) (Figure 3B). Interestingly, we also observed a significant and strong correlation
190 ($p<0.0004$, $r=0.62$) between $CD8^+CXCR5^+$ Tfh cells with $CD19^+$ B cells in LN (Figure 3C). Taken
191 together, the frequencies of $CD4^+CXCR5^+$ and $CD8^+CXCR5^+$ Tfh cells strongly correlate with the
192 frequencies of $CD19^+$ B cells localised within LNs.

193

194

195 **DISCUSSION**

196 We studied $CD4^+$ and $CD8^+$ Tfh cells in both LN biopsies and peripheral blood samples obtained
197 during the earliest phases of RA and compared our findings to control samples. We found an increased
198 frequency of $CD4^+CXCR5^+$ follicular helper T cells and $CD19^+$ B cells in lymphoid tissue of early RA

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199 patients, and CD8$^+$CXCR5$^+$ Tfh cells was similarly increased in LN tissue of both RA-risk individuals
200 and RA patients. This increased frequency of B cells in LNs of RA patients compared to healthy
201 controls is in accordance with previous reports (29, 33). A plausible explanation behind this increase
202 could be the retention of B cells in lymphoid tissue where they eventually differentiate into various B
203 cell effector phenotypes and contribute to disease development. In LN biopsies the frequencies of
204 CD4$^+$CXCR5$^+$ and CD8$^+$CXCR5$^+$ Tfh cells correlate significantly with the frequency of CD19$^+$ B cells
205 suggesting an increased number of B and T cells that can possibly interact at the LN follicular border
206 and drive immune responses. In mice, the location of CD4$^+$ Tfh cells inside the LN during primary and
207 memory responses has been studied in detail (34). During the primary immune response CD4$^+$ Tfh
208 cells are located in the GC follicle, while for a memory response CD4$^+$ Tfh cells are located in the sub
209 capsular region and can leave the follicle via the lymphatic flow. This enables CD4$^+$ Tfh cells to
210 migrate through blood towards other secondary lymphoid organs or inflamed tissues where they can
211 initiate new GC responses if the antigen is present (14). These migrating CD4$^+$ Tfh cells in blood can
212 be present before differentiation towards a fully mature effector Tfh phenotype (35). In our study, the
213 frequency of active and quiescent CD4$^+$ Tfh cells in blood was not significantly altered in RA and RA-
214 risk individuals compared with HCs.

215 CD8$^+$ T cells often function as an effector cytotoxic T cell type hence, they were assumed to be
216 excluded from entry into B cell follicles and participation in GC reactions (36, 37). However, recent
217 data suggest that CD8$^+$ T cells like their CD4$^+$ counterpart are able to acquire CXCR5 which enables
218 their migration into B cell follicles and subsequently eliminate virus infected B cells as well as CD4$^+$
219 Tfh cells (19). Our findings on the presence and increased frequency of CD8$^+$ Tfh cells in lymphoid
220 tissues of early RA patients confirm the presence of CD8$^+$ Tfh cells in lymphoid structures. A study by
221 Kang *et al* was among the first to report the presence of CD8$^+$ T cells in ectopic lymphoid follicles in
222 joints of RA patients (38). In addition, a recent study reported an increased frequency of

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223 $CD8^+CXCR5^+PD1^+$ Tfh cells in LN tissues of IL-2 knock-out mice which were shown to secrete IL-
224 21 and promote B cell antibody class-switching. Interestingly, these $CD8^+$ T follicular cells continued
225 to expand in terms of frequency and numbers over time in these mice (20). Although we did not
226 investigate the B cell class-switching potential of the observed lymph node $CD8^+$ Tfh cells, their
227 frequency correlated with the numbers of B cells. Human studies are needed to further unravel the
228 potential of these cells to participate in a typical GC reaction such as B cell selection.

229 Even though Tfh cells are thought to be crucial for B differentiation within GCs, a recent study
230 identified another type of B-cell promoting T helper cell in peripheral blood and synovial tissue
231 expressing high levels of PD-1 while negative for CXCR5 (26). These cells, identified as peripheral
232 helper cells (Tph), express factors like IL-21, CXCL13 and ICOS enabling them to provide B cell help
233 and drive B cell differentiation. Further characterisation of Tph and Tfh cells in lymphoid tissues as
234 well as inflamed synovial tissue is of interest to understand the mechanisms leading to the formation
235 of secondary and tertiary lymphoid structures and subsequent autoimmune responses.

236 Similar to previous findings of decreased production of pro-inflammatory cytokines upon ex vivo
237 stimulation of LN T cells of early RA patients (39, 40), we found decreased production of IL-21 in LN
238 Tfh cells of early RA patients and in part of the RA-risk individuals. This decreased production of IL-
239 21 by $CD4^+CXCR5^+$ and $CD8^+CXCR5^+$ Tfh cells could be a consequence of prolonged antigen
240 stimulation and exposure *in vivo*, resulting in an exhausted phenotype of these cells (41). Interestingly
241 and in accordance with previous reports (39), this phenomenon of decreased IL-21 production by T
242 cells in our study was only observed in LNs but not in peripheral blood. Our results further highlight
243 the possibility that LNs may offer a unique environment that influences the function and phenotype of
244 T cells resulting in disease development. Therefore, future work aimed at unraveling the possibility of
245 follicular helper T cell exhaustion in lymphoid structures is needed. While IL-21 is described as the
246 most important cytokine derived from Tfh cells to promote B-cell differentiation and proliferation, Tfh

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247 cells also produce other cytokines like IL-4 and IFN-y. Indeed, an in vitro study using sorted
248 CD4$^+$CXCR5$^+$ blood Tfh cells from healthy controls (HCs) and chronic hepatitis C infected patients
249 (HCV) showed that Tfh cells from HCV patients produce less IL-21 but are equally capable of driving
250 *in vitro* B cell proliferation and differentiation into antibody producing cells compared with healthy
251 control cells (42). This indicates that B-cell differentiation is not solely dependent on large amounts of
252 IL-21. Another explanation for low ex vivo IL-21 production may be that primary immune responses
253 are depending on IL-21 but that secondary memory responses of Tfh cells are not (42, 43).

254 Even though the number of participants in our study is limited, the data presented here show for the
255 first time the relation between the number of Tfh cells and B cells inside human LN biopsies and
256 increased frequencies of CD4$^+$ and CD8$^+$ Tfh cells in LN biopsies of early RA patients. Since Tfh may
257 amplify B-cell responses and autoantibody production in lymphoid tissue and CD8$^+$ Tfh are already
258 increased in RA-risk individuals, targeting Tfh cells early could be tested as a new approach to prevent
259 further disease progression during the earliest phases of RA.

260

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269 **Author contributions:**

270 Study conception and design: LGB, DMG, PPT. Acquisition of data: THR, JH, BK, NS, KPL, MM.
271 Analysis and interpretation of data: THR, DCA, PPT, DMG, NdV, LGB. All authors revised the article
272 for important intellectual content and all authors have read and approved the final version of the
273 manuscript. Dr. van Baarsen had full access to all of the data in the study and takes responsibility for
274 the integrity of the data and the accuracy of the data analysis.

275

276 **Conflict of Interest**

277 The authors declare that the research was conducted in the absence of any commercial or financial
278 relationships that could be construed as a potential conflict of interest.

279

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408 **Tables**

Table 1: Baseline characteristics of healthy controls (HCs), RA-risk individuals (RA risk) and early RA patients (Early RA)

	HC **n= 17**	**RA risk** **n=24**	**Early RA** **n= 16**
Sex, female (%)	11 (65)	20 (83)	9 (56)
Age (years)#	38.0 (15.91)	48.0 (12.9)	52.0 (14.1)
IgM-RF positive (n (%))	0 (0)	11 (46)	15 (94)
IgM-RF level (kU/l)*	3.3 (1.0-15.0)	21.0 (6.3-190.3)	312.0 (230.0-405.5)
ACPA positive (n (%))	0 (0)	15 (63)	15 (94)
ACPA level (kAU/l)*	4.0 (2.0-9.0)	45.0 (4.3-176.5)	388.0 (103.5-1529.5)
IgM-RF and ACPA both pos. (n (%))	0 (0)	2 (8)	14 (88)
ESR (mm/h)*	nd	7.5 (2.0-12.0)	13.5 (5.0-27.5)
CRP (mg/l)*	0.9 (0.5-2.3)	1.9 (0.9-3.8)	7.7 (4.5-13.2)
68 TJC (n)*	0 (0)	2.0 (1.0-3.8)	15.0 (9.8-21.8)
66 SJC (n)*	0 (0)	0 (0)	10.0 (5.0-13.3)

*Categorical variables: n (%). Continuous variables; #, mean with standard deviation (SD); *, median with interquatile range (IQR); ACPA, anticitrullinated protein antibodies; nd, not determined; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IgM-RF, IgM rheumatoid factor; 68 TJC, tender joint count of 68 joints; 66 SJC, swollen joint count of 66 joints.*

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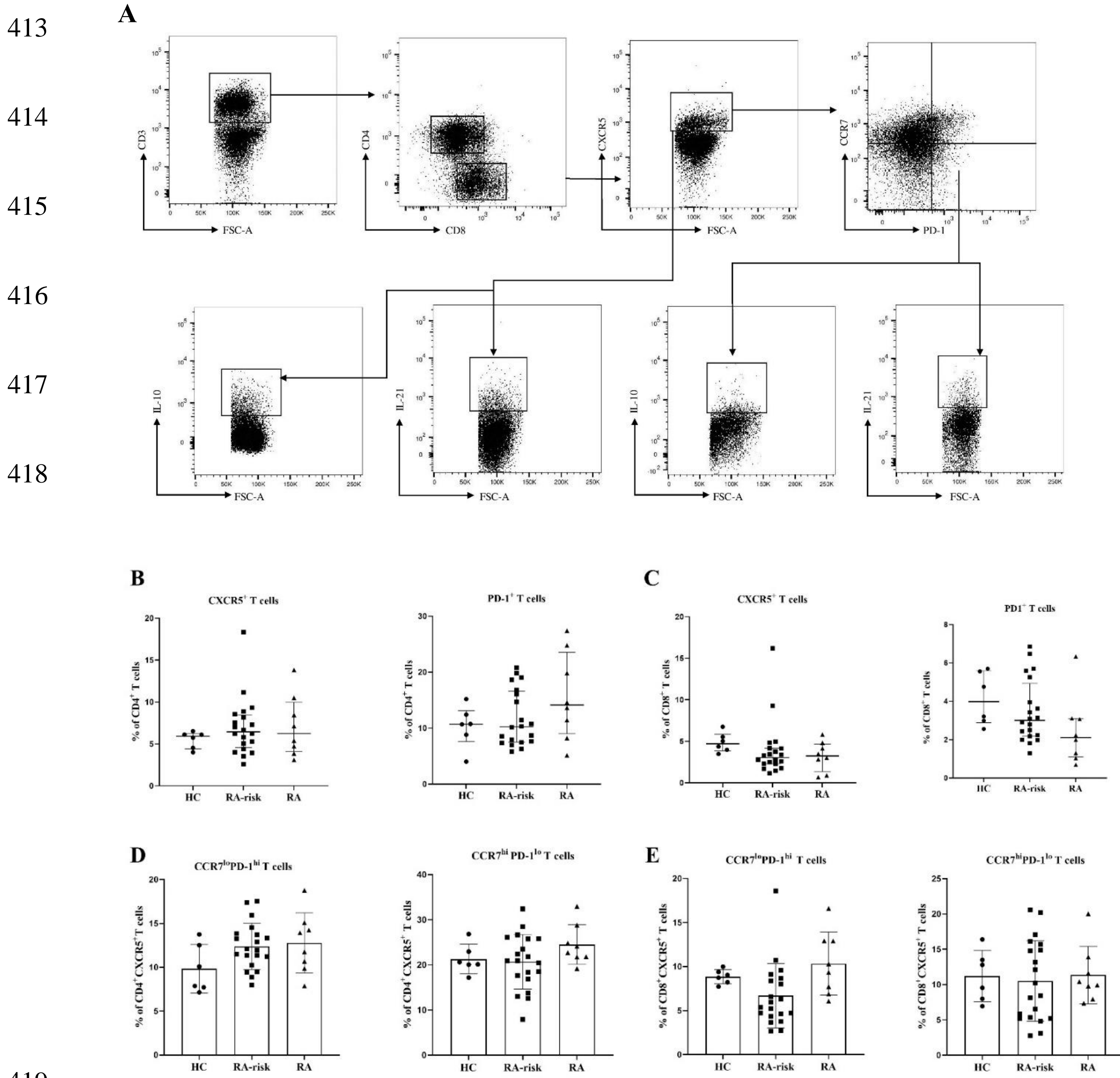
411 **FIGURES**

412 **FIGURE 1**

413



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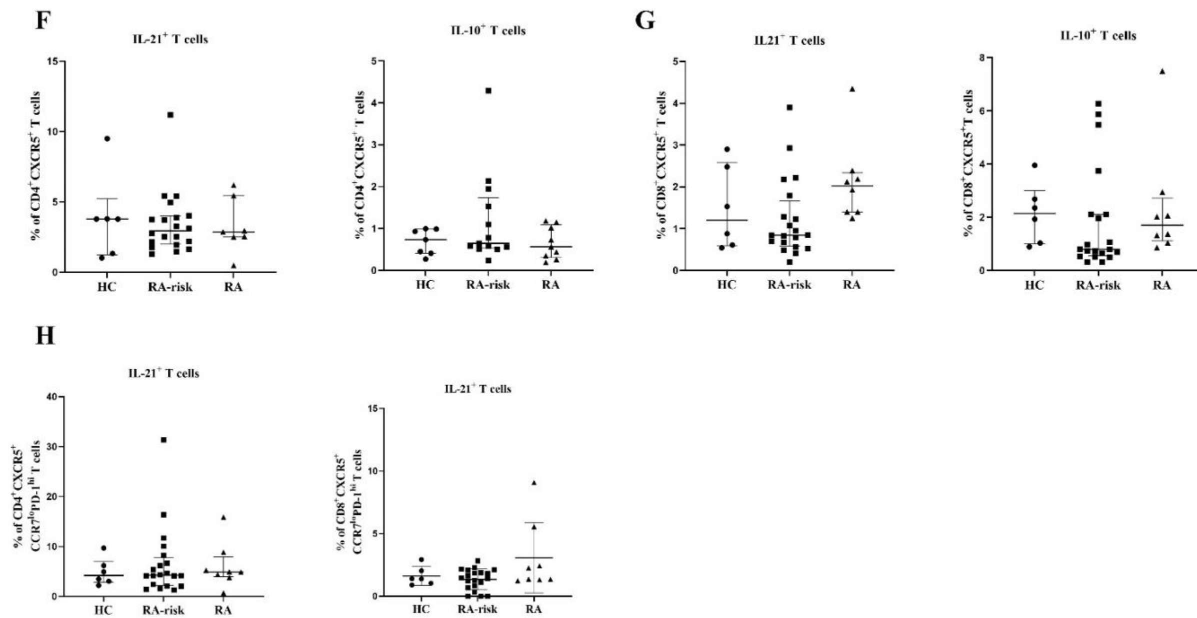

423

424 **Figure 1 | Analysis of circulating follicular helper T cells in peripheral blood samples**. **(A)** Gating
425 strategy for cTfh cells in PBMCs using markers for CD3, CD4, CD8, CXCR5, CCR7, PD-1, IL-21 and
426 IL-10. After gating for single cells, CD4$^+$ and CD8$^+$ T cells were gated within the CD3$^+$ population and
427 further characterized**. (B, C)** The frequencies of CXCR5$^+$ and PD-1$^+$ cells within CD4$^+$ and CD8$^+$ T
428 cells were analysed in PBMC samples collected from healthy controls (HC n=6), RA-risk individuals
429 (RA-risk n=20) and early RA patients (RA n=8). **(D, E)** the frequencies of blood CCR7^{low}PD1^{high} cTfh
430 and CCR7^{high}PD1^{low} cTfh within the CD4$^+$CXCR5$^+$ and CD8$^+$CXCR5$^+$ populations are plotted. **(F, G,**
431 **H)** the frequency of IL-21 and IL-10 producing cells within the indicated T-cell subsets are shown. Not
432 normally distributed data are presented as median with IQR and, normally distributed data are
433 presented in box plots with error bars representing mean with SD. For statistical analysis Kruskall
434 Wallis or one-way ANOVA (when appropriate) was performed. Significant differences were indicated
435 as *p<0.05 or **p<0.01. All symbols represent data from single individuals (• healthy controls (HC),
436 ■ RA-risk individuals (RA-risk), Δ early RA patients (RA).

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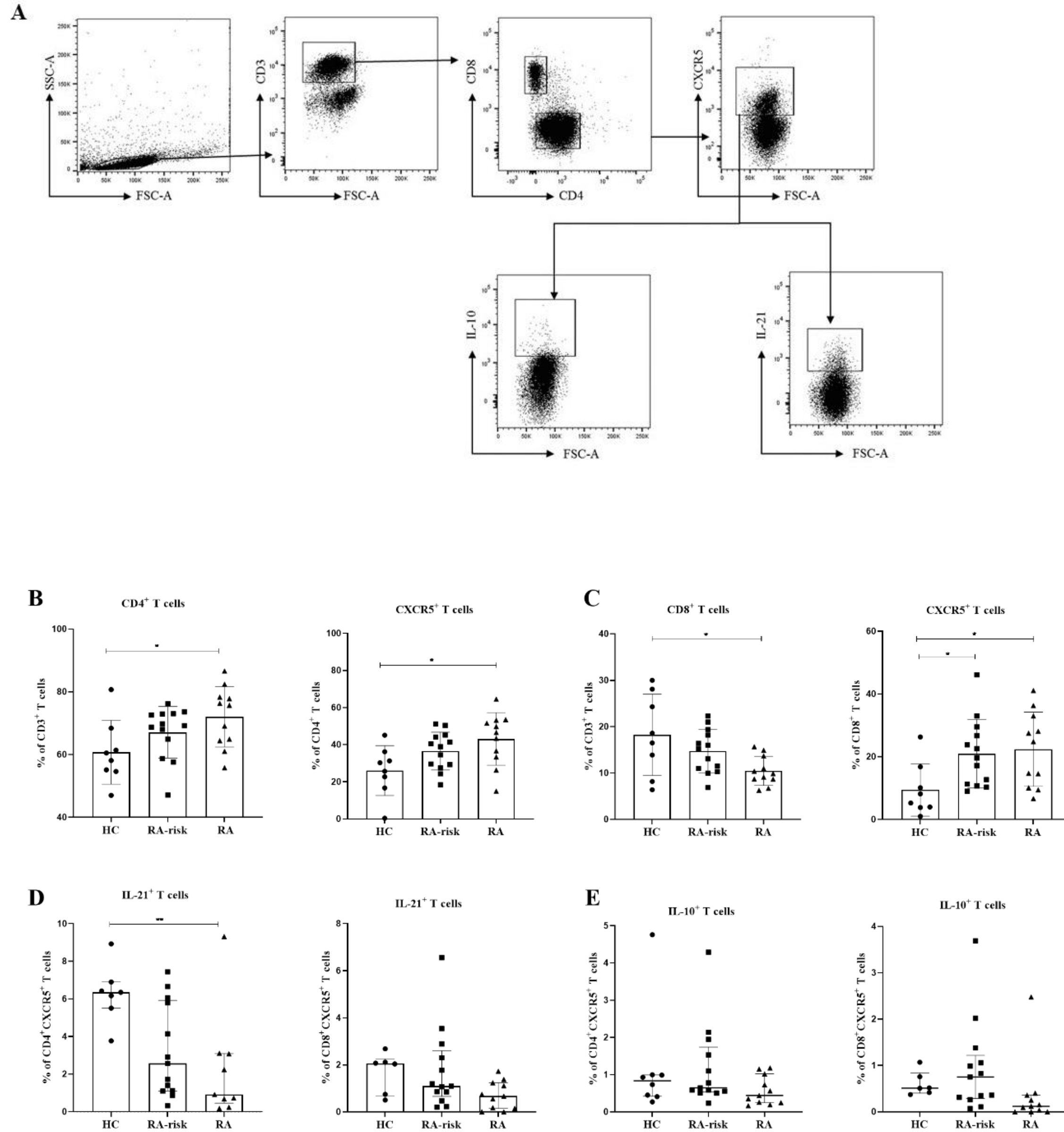
438 **FIGURE 2**



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441 **Figure 2 | Analysis of Follicular helper T cells in lymph node biopsies. (A)** Gating strategy for
442 follicular-like T cells in lymph node biopsies using markers for CD3, CD4, CD8, CXCR5, IL21 and
443 IL-10. **(B, C)** Frequencies of $CD4^+$, $CD4^+CXCR5^+$, $CD8^+$ and $CD8^+CXCR5^+$ T cells in lymph nodes
444 are shown for healthy controls (HC, n=7), RA-risk individuals (RA-risk n=13) and early RA patients
445 (RA n=9). **(D, E)** The frequency of IL-21^+ and IL-10^+cells within the $CD4^+CXCR5^+$ and $CD8^+CXCR5^+$

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446 populations are plotted. Not normally distributed data are presented as median with IQR and, normally
447 distributed data are presented in box plots and error bars represent mean with SD. For statistical
448 analysis Kruskall Wallis or one-way ANOVA (when appropriate) was performed and significant
449 differences were indicated as *p<0.05 or **p<0.01. All symbols represent data from single individuals
450 (• healthy controls (HC), ■ RA-risk individuals (RA at-risk), Δ early RA patients (RA).

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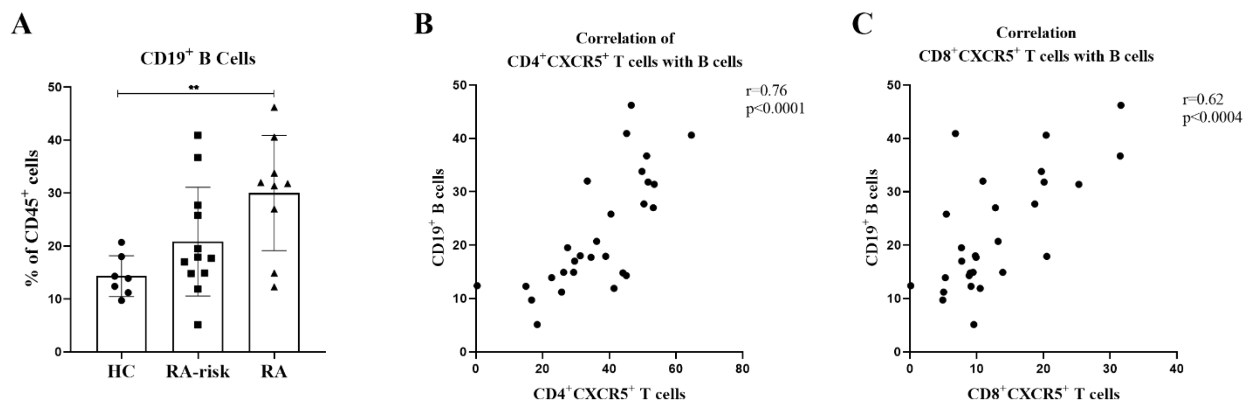
452 **FIGURE 3**



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454 **Figure 3 | Correlation of CD19⁺ B cells with CD4⁺ and CD8⁺ follicular helper T cells in LNs. (A)**
455 frequencies of CD19⁺ B cells in LNs is shown for HCs, RA-risk and early RA patients. **(B)** Correlation
456 between the frequencies of CD4⁺CXCR5⁺ Tfh cells and the frequencies of CD19⁺ B cells in LN. **(C)**
457 Correlation between the frequencies of CD8⁺CXCR5⁺ Tfh cells and the frequencies of CD19⁺ B cells
458 in LNs. Correlations are shown for the total group. Error bars represent mean with SD. For statistical
459 analysis Kruskall Wallis or one-way ANOVA (when appropriate) was performed and significant
460 differences were indicated as *p<0.05 or **p<0.01. All symbols represent data from single individuals
461 (• healthy controls (HC), ■ RA-risk individuals (RA at-risk), Δ early RA patients (RA).

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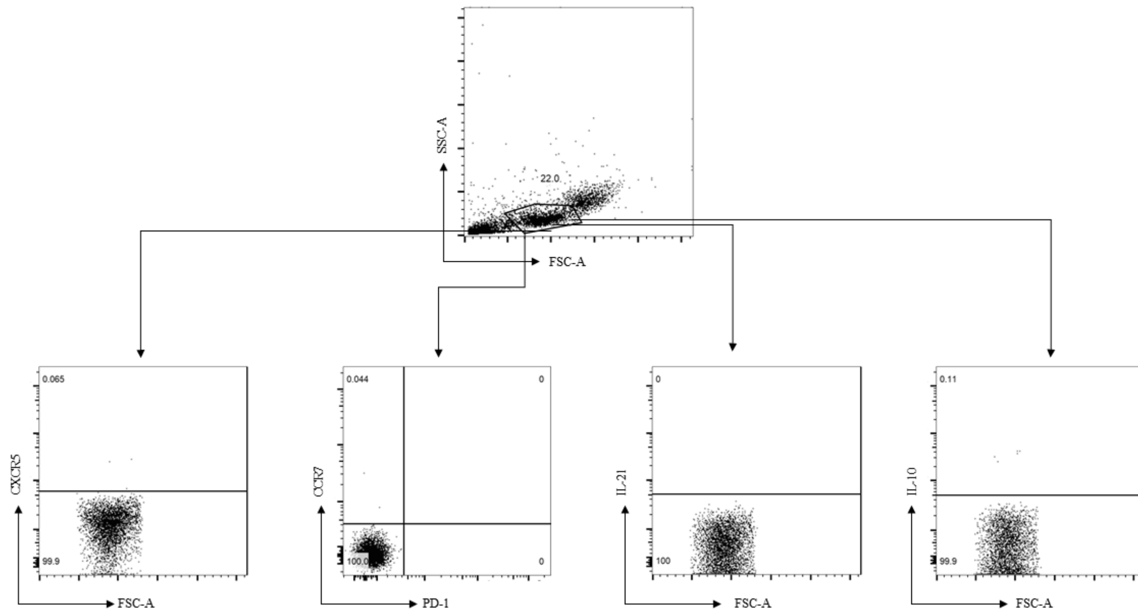
463 **Supplementary Figure 1 |** Negative control (unstained sample) used for setting of gates for the

464 analysis of CXCR5, CCR7, PD-1, IL-21 and IL-10 cells in PBMCs.



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