

1 **CD20<sup>+</sup> T cells have a predominantly Tc1 effector memory phenotype and are**  
2 **expanded in the ascites of patients with ovarian cancer**

3

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16 Short Title:

17 **Expanded CD20<sup>+</sup> T cells in ovarian cancer ascites**

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22

23 **Abbreviations**

24 APC = Antigen-Presenting Cell

25 CTL = Cytotoxic T Lymphocyte

26 FSC = Forward Scatter

27 OC = Ovarian Cancer

28 PBMC = Peripheral Blood Mononuclear Cell

29 SCC = Side Scatter

30 T<sub>C</sub> = Cytotoxic T cell

31 T<sub>CM</sub> = Central Memory T cell

32 T<sub>EM</sub> = Effector Memory T cell

33 T<sub>H</sub> = Helper T cell

34 TIL = Tumor Infiltrating T cell

35 T<sub>Naïve</sub> = Naïve T cell

36 Treg = Regulatory T cell

37 T<sub>TD</sub> = Terminally Differentiated T cell

38

39 **Abstract**

40 Recently, a small subset of T cells that expresses the B cell marker CD20 has been identified in  
41 healthy volunteers and in patients with rheumatoid arthritis and multiple sclerosis. The origin of these  
42 CD20-positive T cells as well as their relevance in human disease remains unclear. Here, we  
43 identified that after functional B cell/T cell interaction CD20 molecules are transferred to the cell  
44 surface of T cells by trogocytosis together with the established trogocytosis marker HLA-DR. Further,  
45 the presence of CD20 on isolated CD20<sup>+</sup> T cells remained stable for up to 48h of *ex vivo* culture.  
46 These CD20<sup>+</sup> T cells almost exclusively produced IFN- $\gamma$  (~70% vs. ~20% in the CD20<sup>-</sup> T cell  
47 population) and were predominantly (CD8<sup>+</sup>) effector memory T cells (~60-70%). This IFN- $\gamma$  producing  
48 and effector memory phenotype was also determined for CD20<sup>+</sup> T cells as detected in the peripheral  
49 blood and ascitic fluids of ovarian cancer patients. In the latter, the percentage of CD20<sup>+</sup> T cells was  
50 further strongly increased (from ~6% in peripheral blood to 23% in ascitic fluid).

51 Taken together, the data presented here indicate that CD20 is transferred to T cells upon intimate T  
52 cell/B cell interaction. Further, CD20<sup>+</sup> T cells are of memory and IFN- $\gamma$  producing phenotype and are  
53 present in increased amounts in ascitic fluid of ovarian cancer patients.

54

55 **Keywords:** CD20, trogocytosis, ovarian cancer, cancer immunology, ascites

56

## 57 **Introduction**

58 Ovarian cancer (OC) remains the most deadly gynecological malignancy with a 5-year survival rate of  
59 only 45%.<sup>1,2</sup> This poor prognosis is largely due to therapy-resistant relapses that occur in the majority  
60 of patients following first line therapy.<sup>3,4</sup> Interestingly, a subset of patients appears to remain disease-  
61 free for prolonged periods of time. To date, several factors have been identified that can be used to  
62 define this particular subset of patients with arguably the strongest prognostic indicator being early  
63 detection and treatment.<sup>3,4</sup> In addition, studies by us and others have revealed strong links between  
64 anti-tumor immune responses and patient survival.<sup>5-8</sup> Specifically, the presence of CD3<sup>+</sup> and CD8<sup>+</sup>  
65 tumor-infiltrating lymphocytes (TIL) and their relative abundance versus regulatory T cells (Treg) is  
66 strongly associated with better survival.<sup>7</sup>

67 In addition to Tumor-infiltrating T cells, it was recently shown that tumor-infiltrating CD20<sup>+</sup> B cells  
68 (CD20<sup>+</sup> TIL) are strongly associated with improved patient survival in high grade serous OC.<sup>9</sup> Of note,  
69 these CD20<sup>+</sup> B-cells were found to strongly co-localize with CD8<sup>+</sup> cytolytic T-cells (CTLs), suggesting  
70 that these cells may work cooperatively to mediate antitumor immunity in OC.<sup>10</sup> In this respect, it is  
71 worth noting that B cells can serve as antigen-presenting cells (APC) to T cells (reviewed in ref. 11).  
72 Indeed, under specific circumstances B-cel APCs can be more effective at antigen presentation than  
73 dendritic cells and may thus contribute to anti-OC immunity by providing local antigen presentation to  
74 T cells.<sup>12</sup>

75 Indirect evidence for such antigen presentation of B to T cells in ovarian carcinoma might be obtained  
76 by evaluating intercellular exchange of membrane components between these two cell types, as  
77 several groups have recently demonstrated that antigen-loaded MHC class II molecules, specifically  
78 HLA-DR, can be transferred between various cells of the immune system during antigen presentation  
79 by a process known as trogocytosis.<sup>13</sup> During trogocytosis, intact proteins, protein complexes and/or  
80 even membrane patches are transferred from one cell type to the other.<sup>14-17</sup> In addition to HLA-DR  
81 transfer, other accessory molecules involved in this contact were found to be similarly transferred.<sup>18</sup>  
82 Interestingly, we and others have recently demonstrated the presence of a small population of T-cells  
83 that express the B-cell marker CD20 in the peripheral blood of healthy volunteers and patients  
84 suffering from rheumatoid arthritis.<sup>19,20</sup> Based on this “hybrid” phenotype, we speculated that these  
85 cells might have acquired B cell membrane molecules during intercellular contact with B cells.

86 Therefore, we here set out to determine whether CD20<sup>+</sup> T cells could originate as a result of B cell/T  
87 cell interaction and whether this population was present in patients with ovarian cancer, in particular in  
88 peripheral blood and in inflammatory ascites fluid.

89

## 90 Results

### 91 T cells acquire CD20 by trogocytosis and maintain a stable CD20-positive phenotype up to 48h 92 after isolation

93 Previous studies by us and others identified a subset of T cells that expressed the typical B cell  
94 marker CD20<sup>19,20</sup>. The origin of such CD20<sup>+</sup> T cells remains unclear, but they may arise as a result of  
95 intercellular membrane exchange during intimate T cell B cell interaction. In line with this, a CD19<sup>-</sup>  
96 CD3<sup>+</sup> CD20<sup>dim</sup> population of T-cells could be clearly identified (Figure 1A and S1A). In a panel of 10  
97 healthy volunteers, this CD20<sup>+</sup> T-cell population comprised 6.0±3.8% of the total T cell population  
98 (Figure 1A). Importantly, the anti-CD20 antibody Rituximab fully specifically blocked CD20 on T cells  
99 as well as on B cells, confirming the specificity of the anti-CD20 antibody we used in identifying the T-  
100 cell subpopulation (Figure S1B). Of note, in CD3<sup>+</sup> T-cells isolated from cord blood this T-cell  
101 population was largely absent (Fig. 1B), which suggests that CD20<sup>+</sup> cells may possibly arise later in  
102 life due to T cell / B cell interaction. Of note, presence of CD20 on the cell surface of CD20<sup>+</sup> T cell  
103 population in the peripheral blood of adult healthy volunteers was stable, with CD20 presence being  
104 retained for up to 48h of culture of isolated CD20<sup>+</sup> T cells (Figure 1C). Reversely, the sorted CD20<sup>-</sup> T-  
105 cell population remained CD20-negative during this time (Figure 1C).

106 To determine whether transfer of CD20 from B cells to T cells could occur by trogocytosis, Jurkat  
107 leukemic T-cells were subsequently mixed with the B cell line Raji. In line with earlier studies, this co-  
108 incubation triggered the rapid transfer of surface HLA-DR to Jurkat T-cells within 15 minutes (Fig. 1D)  
109<sup>13</sup>. Within the same time-frame, CD20 was similarly transferred to Jurkat cells albeit to a lesser extent  
110 (Fig. 1E). Of note, on these Jurkat cells the presence of CD20 was found concurrent with acquisition  
111 of HLA-DR (Fig. 1F). In control monocultures, Jurkat cells did not acquire CD20 or HLA-DR (data not  
112 shown). Thus, CD20 can be transferred from B-cells to leukemic T-cells in a time-frame of minutes.

113 To further evaluate this possible mechanism for peripheral blood T-cells, PBMCs from healthy  
114 volunteers were isolated and mixed with the B cell line Z138, a cell line that expresses high levels of  
115 CD20. After 1h co-culture of PBMCs with Z138, the percentage of primary T cells that expressed  
116 CD20 increased from ~4% to ~30% (Fig. 1G). Similar co-incubation with B-cell Raji also induced  
117 transfer of CD20 to primary T-cells (Fig. 1G, 4% vs. 24%). **Co-incubation of primary T-cells with**  
118 **primary HLA-mismatched B cells was associated with a reproducible increase of ~3% CD20<sup>+</sup> T cells**

119 (Fig. 1G). As reported previously for B cells <sup>15</sup>, trogocytosis was significantly reduced but still  
120 occurred when cells were co-cultured for 1h on ice (not shown). Finally, a small number of CD3<sup>+</sup> T  
121 cells were found to co-express CD20 both in the human tonsil and in lymphoid-like structures in  
122 ovarian tumors (Figure 2A and B, respectively). These CD20<sup>+</sup> T-cells were single cells (Figure 2C)  
123 and had the typical size of T-cells (Figure 2D), with B-cells being significantly larger (Figure 2E).  
124 CD20<sup>+</sup> T-cells were always found in close proximity to B cells and several B cell : T cell pairs  
125 displayed an intimate membrane interaction (Figure 2A).

126

### 127 **CD20<sup>+</sup> T cells are HLA-DR<sup>+</sup> and CCR7/CD45RO<sup>+</sup> effector memory T cells in healthy volunteers** 128 **and ovarian cancer (OC) patients**

129 The above data indicate that T-cells acquire CD20 molecules after B/T cell interaction, which  
130 suggests that CD20 should predominantly be found on memory T-cells. Therefore, the phenotype of  
131 circulating CD20<sup>+</sup> T cells in peripheral blood from healthy volunteers and OC patients was further  
132 determined. CD20 within the T cell population was almost exclusively detected on effector memory T  
133 cells (T<sub>EM</sub>) in both healthy volunteers and OC patients (Figure 3A; 71.4±1.6% and Figure 5A;  
134 63.2±9.0%), as well as on terminally differentiated T cells (T<sub>TD</sub>, Figure 3A; 20.2±2.0% and Figure 5A;  
135 29.7±8.2%). Within this CD20<sup>+</sup> T<sub>EM</sub> population, expression was skewed towards CD8<sup>+</sup> cells over CD4<sup>+</sup>  
136 cells (Figure 3B; 60% vs. 40%). In contrast, CD20<sup>-</sup> T cells displayed a distribution typically found in  
137 peripheral blood with naïve (30.9±9.6%), central memory (T<sub>CM</sub>; 9.1±2.4%), effector memory (T<sub>EM</sub>;  
138 39.9±1.6%) and terminally differentiated T-cells (T<sub>TD</sub>; 20.1±8.8%). Furthermore, CD20<sup>-</sup> T cells  
139 contained a higher percentage of CD4<sup>+</sup> T-cells than CD8<sup>+</sup> T-cells (Figure S1C; 40% vs. 60%).  
140 Interestingly, CD20<sup>+</sup> but not CD20<sup>-</sup> T-cells also expressed HLA-DR on their cell surface (Figure 3C  
141 and S1D), consistent with the possible acquisition of cell membrane from antigen-presenting cells  
142 (APCs). Both CD20<sup>+</sup> and CD20<sup>-</sup> T cells expressed CD127 (IL-7R), but not CD25 (IL-2R) (Figure 3C  
143 and S1D).

144

### 145 **CD20<sup>+</sup> T cells are IFN-γ producing T<sub>H</sub>1/T<sub>C</sub>1 cells in healthy volunteers and OC patients**

146 To further characterize the CD20<sup>+</sup> T cell population, intracellular cytokine stainings were performed to  
147 identify T<sub>H</sub>1/T<sub>C</sub>1, T<sub>H</sub>2/T<sub>C</sub>2 or T<sub>H</sub>17 cells. CD3<sup>+</sup> PBMCs were stimulated with PMA/ionomycin in the  
148 presence of Brefeldin A and intracellular cytokine staining performed in conjunction with CD19, CD20

149 and CD45RO. As anticipated, non-gated CD3<sup>+</sup> T cells as well as the CD20<sup>-</sup> T-cell population  
150 contained both IFN- $\gamma$  (Figure 4A), IL-4 (Figure 4B) and IL-17 (Figure 4C) producing cells, with  
151 CD45RO<sup>+</sup> memory T cells being largely responsible for cytokine secretion (Figure 4A-C; left panels).  
152 However, within the CD20<sup>+</sup> T cell population, that consisted of >95% CD45RO<sup>+</sup> memory T-cells,  
153 almost all cells produced IFN- $\gamma$ , with only a small percentage of cells that produced IL-4 and no IL-17  
154 production (Figure 4A-C). These findings were subsequently verified using multi-color fluorescent  
155 microscopy (Figure S2A). **Of note, 4h stimulation with PMA/ionomycin did not shift CD45RA to**  
156 **CD45RO cells in these experiments (Figure S2B)**

157

### 158 **The percentage of CD20<sup>+</sup> T cells is increased in ascites fluid of ovarian cancer patients**

159 Patients with ovarian cancer had CD20<sup>+</sup> T cell populations in the peripheral blood that closely  
160 matched that found in healthy volunteers, including a predominant T<sub>H</sub>1/T<sub>C</sub>1 T<sub>EM</sub> phenotype (Figure  
161 5A), with perhaps a minor trend towards a more T<sub>TD</sub> phenotype (Figure 5D). However, in peritoneal  
162 ascites fluid of OC patients the population of CD20<sup>+</sup> T cells was significantly expanded and comprised  
163 approximately 23.4 $\pm$ 6.8% of all ascites fluid T cells (Figure 5B; left panel and Figure 5C). These  
164 CD20<sup>+</sup> ascites fluid T-cells were predominantly of T<sub>EM</sub> phenotype (Figure 5B and D) and were also  
165 skewed towards the T<sub>H</sub>1/T<sub>C</sub>1 cytokine production (Figure S3A-C).

166



## 167 Discussion

168 In the present study, we demonstrate that T cells rapidly acquired CD20 (<15 min.) when co-cultured  
169 *in vitro* with B cell leukemic lines or primary B-cells. In peripheral blood and in ascites fluid these  
170 CD20+ T cells were phenotypically characterized as effector memory T-cells that produced IFN- $\gamma$ .  
171 Further, this CD20<sup>+</sup> T-cell population was significantly enriched in ascites fluid in ovarian cancer  
172 patients.

173 Data from the current study suggests that CD20<sup>+</sup> T cells may arise as a result of membrane exchange  
174 upon T cell : B cell contact. These findings are consistent with the previously reported transfer of HLA-  
175 DR from B cells to T cells<sup>13</sup>. Concurrent with uptake of CD20 a similar uptake of HLA-DR by T cells  
176 was detected. It is therefore conceivable that CD20<sup>+</sup> T cells originate following antigen-presentation  
177 by B cells and concomitant transfer of both HLA-DR and CD20 (and possibly other molecules).  
178 Indeed, the absence of CD20<sup>+</sup> T cells in cord blood seems to support a role for the development of  
179 CD20<sup>+</sup> T cells during immune responses later in life.

180 In our cohort of healthy volunteers and OC patients (>30 individuals in total), approximately 2-10%  
181 (6.0 $\pm$ 3.8%) of circulating T cells in the peripheral blood express CD20 on the T cell surface.  
182 Furthermore, multiple samplings of the same healthy volunteer in a 2 week interval revealed highly  
183 consistent levels of this subpopulation (data not shown). These findings are in line with other recent  
184 reports on the relative percentage of CD3<sup>+</sup> CD20<sup>+</sup> T cells in the peripheral blood of German and  
185 British cohorts of healthy volunteers and patients with rheumatoid arthritis<sup>19,20</sup>.

186 Importantly, as CD20 is considered a prototypical B cell marker, studies on CD20<sup>+</sup> T cells should take  
187 exceptional care to exclude any contamination by B cell-T cell doublets. Indeed, Henry et al. have  
188 previously suggested that CD20<sup>+</sup> T cells in peripheral blood may be an artifact of flow cytometry  
189 resulting from doublets.<sup>21</sup> However, we and others have demonstrated that single isolated T cells can  
190 and do have CD20 molecules at their cell surface.<sup>20</sup> Nevertheless, we addressed these concerns  
191 further in our current study in several ways. First, we have used a highly stringent gating strategy to  
192 exclude not only doublets (forward scatter pulse width area), but also B cells based on the co-  
193 expression of CD19. Second, analysis of CD20<sup>+</sup> T cell phenotype by confocal microscopy did not  
194 reveal any signs of B cell-T cell doublets, whereas we could clearly identify CD3<sup>+</sup> CD20<sup>+</sup> cells with  
195 CD20 levels distinct from a B cell (~10-100 fold lower). Third, CD3<sup>+</sup> CD20<sup>+</sup> T cells could be isolated

196 from peripheral blood by single cell sorting, activated with a cocktail of anti-CD3 and IL-2 and  
197 remained a single homogenous population presenting CD20 and expressing typical T cell markers.  
198 Fourth, cord blood T cells were largely devoid of a CD3<sup>+</sup> CD20<sup>+</sup> T cells under identical staining  
199 conditions. Fifth, B cell contamination in our phenotypical analysis should have resulted in a distinct  
200 population with characteristic B cell expression levels for CD45RO/CCR7/CD25/CD127/ HLA-DR, but  
201 no such population was observed. Therefore, we are confident of and support Wilk et al. on the  
202 validity of CD3<sup>+</sup> CD20<sup>+</sup> T cells in peripheral blood.<sup>19</sup>

203 CD20<sup>+</sup> T cells from peripheral blood were found to display a typical effector memory (T<sub>EM</sub>) phenotype  
204 and were skewed towards a CD8<sup>+</sup> (~60%) Th1/Tc1 subtype. Furthermore, when performing  
205 phenotypical analysis of the CD20<sup>+</sup> T cell subpopulation in the peripheral blood or ascites fluid of  
206 patients with ovarian cancer, we found that this phenotype was fully conserved within individuals with  
207 ovarian cancer. Of note, while Wilk et al. primarily examined T cell markers associated with activation  
208 status, their observed percentage of CD45RO expressing cells in CD20<sup>+</sup> vs. CD20<sup>-</sup> cells is almost  
209 identical to the one observed by us (Wilk et al. 72% vs. 42%; this study 76.7±1.9% vs. 49±2.4%)<sup>19,23</sup>.

210 As CD20<sup>+</sup> T cells are predominantly T<sub>EM</sub> cells with a Tc1 (IFN-γ<sup>+</sup> CD8<sup>+</sup>) phenotype, it is tempting to  
211 speculate that these cells function as a tumor suppressor population. Indeed, infiltration of T<sub>EM</sub> cells  
212 into ovarian cancer tumors has been correlated to improved disease progression and Tc1 cells have  
213 been extensively described as the main mediators of the anti-tumor T cell response.<sup>6</sup> However, the  
214 relative levels of CD20<sup>+</sup> T cells in ascites fluid were highly consistent and did not appear to correlate  
215 to disease stage, therapy response or expected prognosis. Alternatively, T<sub>EM</sub> cells and by extension  
216 CD20<sup>+</sup> T cells might be expanded in the peritoneal cavity as a result of increased homing to peripheral  
217 tissues consistent with the function of T<sub>EM</sub> cells versus central memory T cells.

218 One outstanding question that remains is whether there are functional differences between CD20<sup>+</sup>  
219 and CD20<sup>-</sup> T-cells as a consequence of CD20 expression on the T-cell surface. On B-cells, CD20  
220 ligation by agonistic antibodies was reported to induce intracellular calcium fluxes and thereby to  
221 augment B-cell receptor signaling<sup>24-26</sup>. However, the natural ligand of CD20 is currently unknown and  
222 whether calcium signaling is the primary signal for CD20 in its natural context remains to be  
223 determined. In this respect, B cells from a juvenile patient with CD20 deficiency did not differ in basal  
224 calcium flux in response to treatment with IgG or IgM, but were defective in antibody production<sup>27</sup>.  
225 Further insight into the role of CD20 may help uncover whether CD20 has a function on CD20<sup>+</sup> T-

226 cells.

227 In conclusion, we describe here the in depth characterization of CD20<sup>+</sup> T cells from peripheral blood  
228 as CD8<sup>+</sup> effector memory and IFN- $\gamma$  producing T-cells. Further, we document a significant expansion  
229 of this population in the ascites fluid of patients with ovarian cancer and provide insights into the  
230 possible origin of these cells. Further studies should aim to elucidate whether CD20<sup>+</sup> T cells occur *de*  
231 *novo* as a result of B cell antigen presentation in patients with OC and whether this T-cell population  
232 is involved in antigen-specific immunity against OC.

233

234 **Materials & Methods**

235 **Antibodies and reagents**

236 Anti-CD3-CyQ, anti-CD3-PE, anti-CD4-PE, anti-CD8-PE, anti-CD19-PE, anti-CD20-FITC and anti-  
237 HLA-DR-PE were from IQ products (Groningen, The Netherlands). Anti-CD45RO-APC, anti-CCR7-  
238 PerCP-Cy5.5, anti-CD25-PE, anti-CD127-APC, anti-IFN $\gamma$ -PerCP-Cy5.5, anti-IL-4-PerCP-Cy5.5 and  
239 anti-IL-17A-PerCP-Cy5.5 were from eBioscience (San Diego, CA). Fluorescently-conjugated isotype  
240 controls for each antibody were ordered from the same companies as indicated above. The agonistic  
241 anti-CD3 antibody WT-32 was kindly provided by Dr. B.J. Kroesen (University of Groningen, The  
242 Netherlands). IL-2 was purchased from immunotools (Friesoythe, Germany).

243

244 **Cell lines and trogocytosis assays**

245 The T cell line Jurkat and B cell lines Z138 and Raji were purchased from the ATCC. Trogocytosis  
246 was assessed by co-culturing Jurkat or primary T cells with Z138 or Raji for various time points as  
247 indicated, followed by flow cytometric analysis of cell surface markers as described below.

248

249 **Isolation and activation of primary (patient-derived) immune cells**

250 Experiments were approved by the local Medical Ethical Committee and patients/healthy volunteers  
251 signed for informed consent. Peripheral blood lymphocytes (PBL) from blood of healthy donors or  
252 cancer patients were isolated using standard density gradient centrifugation (Lymphoprep; Axis-  
253 Shield PoC As) as previously described.<sup>22</sup> Tumor-associated immune cells were isolated from the  
254 primary ascites cultures using ammonium chloride lysis. Activated T cells were generated by culturing  
255 PBLs with anti-CD3 mAb WT-32 (0.5  $\mu$ g/mL) and IL-2 (100 ng/mL) for 48 hours. Cord blood cells  
256 (following CD34<sup>+</sup> depletion by magnetic cell sorting) were kindly provided by Prof. Dr. J.J. Schuringa  
257 (University of Groningen, The Netherlands)

258

259 **Cell surface immunofluorescence staining**

260 For determining the percentage of CD20<sup>+</sup> T cells, 0.5x10<sup>6</sup> cells per indicated condition were stained  
261 with anti-CD3-CyQ, anti-CD19-PE and anti-CD20-FITC. For phenotypic characterization of T cells,  
262 0.5x10<sup>6</sup> cells per indicated condition were stained with anti-CD3-PE (or alternatively anti-CD4-PE or

263 anti-CD8-PE), anti-CD20-FITC, anti-CD45RO-APC and anti-CCR7-PerCP-Cy5.5. Expression of IL-  
264 2R $\alpha$ , IL-7R $\alpha$  or HLA-DR was determined by staining  $0.5 \times 10^6$  cells per indicated condition with anti-  
265 CD3-PerCP-Cy5.5, anti-CD20-FITC, anti-CD25-PE and anti-CD127-APC. All staining was carried out  
266 for 60 minutes on ice in the dark and specific staining of all indicated markers was confirmed using  
267 relevant isotype controls. Staining was analyzed on a BD Accuri C6 flow cytometre (Becton  
268 Dickinson). Data was plotted using Cflow software (Becton Dickinson). Positively and negatively  
269 stained populations were calculated by quadrant dot plot analysis. For all experiments, cells were  
270 carefully gated on forward scatter pulse width area to exclude doublets and B cells excluded from the  
271 analysis by co-expression of CD19.

272

### 273 **Intracellular immunofluorescence staining**

274 Immune cells were washed and stimulated with PMA/Ionomycin in the presence of brefeldin A for 4h.  
275 Subsequently, cells were washed in wash buffer (phosphate buffered saline, 5% fetal bovine serum,  
276 0.1% sodium azide) and stained with PE-conjugated anti-CD3, FITC-conjugated anti-CD20 and APC-  
277 conjugated anti-CD45RO for 45 minutes on ice. Cells were subsequently fixed with Reagent A  
278 (Caltag, An Der Grab, Austria) for 10 minutes. After washing, cells were resuspended in  
279 permeabilization Reagent B (Caltag) and labeled with either anti-IFN $\gamma$ , anti-IL-4 or anti-IL-17A  
280 antibodies conjugated to PerCP-Cy5.5 for 20 minutes in the dark. Relevant isotype-matched  
281 antibodies were used as controls. After staining, the cells were washed and analyzed on a BD Accuri  
282 C6 flow cytometre (Becton Dickinson). Data was plotted using Cflow software (Becton Dickinson).  
283 Positively and negatively stained populations were calculated by quadrant dot plot analysis.

284

### 285 **Two-photon confocal microscopy**

286 Immune cells were stained essentially as described above for intracellular immunofluorescence with  
287 the exception that no anti-CD45RO-APC antibody was added during the initial cell surface staining  
288 after stimulation with PMA/Ionomycin. Cells were subsequently analyzed on an inverted LSM 780  
289 NLO Zeiss microscope (Axio Observer.Z1) with the kind help of Ing. K.A. Sjollem.

290

### 291 **Multi-color immunofluorescence on paraffin-embedded tissue**

292 Tonsil and tumor slides were deparaffinized, rehydrated, and antigen retrieval was performed in a  
293 citrate buffer (10 mM citrate, pH 6.0). After cooling, endogenous peroxidase was blocked in a 0.3%  
294 H<sub>2</sub>O<sub>2</sub> solution for 30 minutes. Slides were then incubated overnight with rat anti-human CD3 (Abcam,  
295 ab5690, 1:20) and mouse anti-human CD20 (DAKO, clone L26, 1:100). CD3 signal was visualized  
296 using a HRP-conjugated goat-anti-rat secondary antibody and Cy5 tyramide signal amplification  
297 according to the manufacturer's instructions (PerkinElmer). CD20 was visualized using  
298 AlexaFluor488-conjugated goat anti-mouse secondary antibody. Counterstaining was done by 4',6-  
299 diamidino-2-phenylindole (DAPI). Slides were mounted in Prolong Gold (Life Technologies) and  
300 stored in the dark at RT. Immunofluorescent slides were scanned using a TissueFAXS imaging system  
301 (TissueGnostics, Austria). Processed channels were merged using Adobe Photoshop.

302

### 303 **Statistical analysis**

304 Data reported are mean values  $\pm$  SD of at least three independent experiments. Statistical analysis  
305 was performed by one-way ANOVA followed by Tukey-Kramer post test or, where appropriate, by  
306 two-sided unpaired Student's t-test.  $p < 0.05$  was defined as a statistically significant difference.  
307 Where indicated \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

308

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313

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- 397

398 **Figure legends**

399 **Figure 1. T cells can acquire CD20 upon co-culture with B cells.** **A-B** Peripheral blood of healthy  
400 volunteers (A) or cord blood (B) was stained using anti-CD3-CyQ, anti-CD19-PE and anti-CD20-FITC.  
401 Plots represent cells gated on FSC/SSC followed by exclusion of B-cells based on co-expression of  
402 CD19 and CD20. **C** PBMCs from healthy volunteers were stained using anti-CD3-CyQ, anti-CD19-PE  
403 and anti-CD20-FITC and CD20<sup>-</sup> and CD20<sup>+</sup> T cell populations isolated using multicolor cell sorting.  
404 Isolated cells were left untreated or activated using a cocktail of anti-CD3 mAb and IL-2 for 48h after  
405 which cells were examined for CD3 and CD20 expression. **D-F** Jurkat T cells were co-cultured with  
406 CD20<sup>+</sup> B cell line Raji for 15 minutes and expression of HLA-DR (D), CD20 (E) or both (F) was  
407 assessed by flow cytometry on gated CD3<sup>+</sup> T-cells. **G** PBMCs of healthy volunteers were incubated  
408 alone or in the presence of Z138, Raji or healthy control B cells at a ratio of 2-1 for 1h followed by flow  
409 cytometric analysis for CD20 expression within the CD3<sup>+</sup> population. Asterisks represent significant  
410 changes compared to PBMCs alone.

411  
412 **Figure 2. *In situ* identification of CD3<sup>+</sup> CD20<sup>+</sup> T-cells.** **A** Tonsil and **B** ovarian tumor tissue was  
413 stained for CD3 and CD20 and co-expression assessed by multicolor immunofluorescent microscopy.  
414 Insets identify the region where individual CD3<sup>+</sup> CD20<sup>+</sup> T cells could be identified. **C** single cell  
415 expression of CD3 and CD20 was validated using counterstaining with DAPI. **D** CD20<sup>-</sup> CD3<sup>+</sup> T-cells  
416 and **E** CD20<sup>+</sup> CD3<sup>-</sup> B cells could also be readily identified.

417  
418 **Figure 3. Phenotype of CD20<sup>+</sup> T-cells.** **A** PBMCs from healthy volunteers were stained using anti-  
419 CD3-CyQ, anti-CD19-PE and anti-CD20-FITC and prevalence of T cell subpopulations assessed  
420 using flow cytometry. PBMCs were strictly gated on FSC/SSC to exclude B/T doublets (left panel) and  
421 B cells excluded from analysis after identification based on co-expression of CD19 and CD20 (middle  
422 panel). **B** PBMCs from healthy volunteers were stained using anti-CD3-PE, anti-CD20-FITC, anti-  
423 CD45RO-APC and anti-CCR7-PerCP-Cy5.5 mAbs and analyzed using flow cytometry. **C** PBMCs  
424 from healthy volunteers were stained using anti-CD20-FITC, anti-CD45RO-APC and anti-CCR7-  
425 PerCP-Cy5.5 (left panel) in combination with either anti-CD4-PE (middle panel) or anti-CD8-PE (right  
426 panel) mAbs and analyzed using flow cytometry. Middle and right panels are gated on the CD20<sup>+</sup> T  
427 cells. **D** CD20<sup>+</sup> T cells identified as described in A were further characterized for expression of CD25

428 (left panel), CD127 (middle panel) or HLA-DR (right panel) using flow cytometry. Percentages±SD are  
429 representative of 7 healthy donors.

430

431 **Figure 4. Cytokine production by CD20<sup>+</sup> T cells.** PBMCs from healthy volunteers were stimulated  
432 for 4h using PMA/Ionomycin in the presence of Brefeldin A and intracellular production of IFN-γ (**A**),  
433 IL-4 (**B**) and IL-17A (**C**) assessed in the CD3<sup>+</sup> CD20<sup>-</sup> and CD3<sup>+</sup> CD20<sup>+</sup> populations assessed using  
434 flow cytometry. Anti-CD45RO-APC was included to identify memory T cell populations.

435

436 **Figure 5. Prevalence and subtype of CD20<sup>+</sup> T-cells in patients with ovarian cancer.** PBMCs (**A**)  
437 or ascites fluid cells (**B**) from patients with ovarian cancer were stained using anti-CD3-PE, anti-  
438 CD20-FITC, anti-CD45RO-APC and anti-CCR7-PerCP-Cy5.5 mAbs and analyzed using flow  
439 cytometry. **C-D** Percentages (**C**) and subset distribution (**D**) of CD20<sup>+</sup> T cells in peripheral blood of  
440 healthy volunteers, peripheral blood of patients with ovarian cancer and in ascites fluid of ovarian  
441 cancer. Data were analyzed as described in the legend to Figure 1.

442

443 **Supplementary Figure legends**

444 **Figure S1. Phenotype of CD20<sup>+</sup> T-cells.** **A** PBMCs from healthy volunteers were stained using anti-  
445 CD3-CyQ, anti-CD19-PE and anti-CD20-FITC (left panel) or anti-CD3-CyQ, FITC-labeled isotype  
446 control and PE-labeled isotype control (middle and right panel) and analyzed using flow cytometry.  
447 PBMCs were strictly gated on FSC/SSC to exclude B/T doublets (Figure 3; left panel). **B** PBMCs from  
448 healthy volunteers were left untreated or treated on ice for 45 minutes with anti-CD20 antibody  
449 Rituximab followed by staining using using anti-CD3-CyQ, anti-CD19-PE and anti-CD20-FITC mAbs  
450 and analyzed using flow cytometry. **C** PBMCs from healthy volunteers were stained using anti-CD20-  
451 FITC, anti-CD45RO-APC and anti-CCR7-PerCP-Cy5.5 (left panel) in combination with either anti-  
452 CD4-PE (middle panel) or anti-CD8-PE (right panel) mAbs and analyzed using flow cytometry. Middle  
453 and right panels are gated on the CD20<sup>-</sup> T cells. **D** CD20<sup>-</sup> T cells identified as described in Figure 3A  
454 were further characterized for expression of CD25 (left panel), CD127 (middle panel) or HLA-DR (right  
455 panel) using flow cytometry. Percentages±SD are representative of 7 healthy donors.

456

457 **Figure S2. Phenotype and cytokine production of CD20<sup>+</sup> T-cells.** **A** PBMCs from healthy  
458 volunteers were stained for CD3, CD20 and IFN-γ and analyzed using confocal microscopy. **B**  
459 PBMCs from healthy volunteers were stimulated for 4h using PMA/Ionomycin in the presence of  
460 Brefeldin A or left untreated, and expression of CD45RO within the CD3<sup>+</sup> population assessed by flow  
461 cytometry.

462

463 **Figure S3. Cytokine production by CD20<sup>+</sup> T cells from ascites fluid.** Cells from ascites fluid  
464 derived from a patient with ovarian cancer were stimulated for 4h using PMA/Ionomycin in the  
465 presence of Brefeldin A and intracellular production of IFN-γ (**A**), IL-4 (**B**) and IL-17A (**C**) assessed in  
466 the CD3<sup>+</sup> CD20<sup>-</sup> and CD3<sup>+</sup> CD20<sup>+</sup> populations assessed using flow cytometry. Anti-CD45RO-APC  
467 was included to identify memory T cell populations.