1 **CD20^{+} T cells have a predominantly Tc1 effector memory phenotype and are**

2 **expanded in the ascites of patients with ovarian cancer**

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16 Short Title:

17 **Expanded CD20^{+} T cells in ovarian cancer ascites**

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Abbreviations

APC = Antigen-Presenting Cell

CTL = Cytotoxic T Lymphocyte

FSC = Forward Scatter

OC = Ovarian Cancer

PBMC = Peripheral Blood Mononuclear Cell

SCC = Side Scatter

T_C = Cytotoxic T cell

T_{CM} = Central Memory T cell

T_{EM} = Effector Memory T cell

T_H = Helper T cell

TIL = Tumor Infiltrating T cell

$T_{Naïve}$ = Naïve T cell

Treg = Regulatory T cell

T_{TD} = Terminally Differentiated T cell

Abstract

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

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

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

Introduction

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

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

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

4

86   Therefore, we here set out to determine whether CD20$^+$ 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

Results

T cells acquire CD20 by trogocytosis and maintain a stable CD20-positive phenotype up to 48h

after isolation

Previous studies by us and others identified a subset of T cells that expressed the typical B cell marker CD20 [19,20]. The origin of such CD20+ T cells remains unclear, but they may arise as a result of intercellular membrane exchange during intimate T cell B cell interaction. In line with this, a CD19- CD3+ CD20dim population of T-cells could be clearly identified (Figure 1A and S1A). In a panel of 10 healthy volunteers, this CD20+ T-cell population comprised 6.0±3.8% of the total T cell population (Figure 1A). Importantly, the anti-CD20 antibody Rituximab fully specifically blocked CD20 on T cells as well as on B cells, confirming the specificity of the anti-CD20 antibody we used in identifying the T-cell subpopulation (Figure S1B). Of note, in CD3+ T-cells isolated from cord blood this T-cell population was largely absent (Fig. 1B), which suggests that CD20+ cells may possibly arise later in life due to T cell / B cell interaction. Of note, presence of CD20 on the cell surface of CD20+ T celll population in the peripheral blood of adult healthy volunteers was stable, with CD20 presence being retained for up to 48h of culture of isolated CD20+ T cells (Figure 1C). Reversely, the sorted CD20- T-cell population remained CD20-negative during this time (Figure 1C).

To determine whether transfer of CD20 from B cells to T cells could occur by trogocytosis, Jurkat leukemic T-cells were subsequently mixed with the B cell line Raji. In line with earlier studies, this co-incubation triggered the rapid transfer of surface HLA-DR to Jurkat T-cells within 15 minutes (Fig. 1D) [13]. Within the same time-frame, CD20 was similarly transferred to Jurkat cells albeit to a lesser extent (Fig. 1E). Of note, on these Jurkat cells the presence of CD20 was found concurrent with acquisition of HLA-DR (Fig. 1F). In control monocultures, Jurkat cells did not acquire CD20 or HLA-DR (data no shown). Thus, CD20 can be transferred from B-cells to leukemic T-cells in a time-frame of minutes.

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

119 (Fig. 1G). As reported previously for B cells [15], trogocytosis was significantly reduced but still

120 occurred when cells were co-cultured for 1h on ice (not shown). Finally, a small number of $CD3^+$ 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 $CD20^+$ 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 $CD20^+$ 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 **$CD20^+$ T cells are HLA-DR$^+$ and CCR7$^-$/CD45RO$^+$ 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 $CD20^+$ 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_{EM}) 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_{TD}, Figure 3A; 20.2±2.0% and Figure 5A;

135 29.7±8.2%). Within this $CD20^+$ T_{EM} population, expression was skewed towards $CD8^+$ cells over $CD4^+$

136 cells (Figure 3B; 60% vs. 40%). In contrast, $CD20^-$ T cells displayed a distribution typically found in

137 peripheral blood with naïve (30.9±9.6%), central memory (T_{CM}; 9.1±2.4%), effector memory (T_{EM};

138 39.9±1.6%) and terminally differentiated T-cells (T_{TD}; 20.1±8.8%). Furthermore, $CD20^-$ T cells

139 contained a higher percentage of $CD4^+$ T-cells than $CD8^+$ T-cells (Figure S1C; 40% vs. 60%).

140 Interestingly, $CD20^+$ but not $CD20^-$ 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 $CD20^+$ and $CD20^-$ T cells expressed CD127 (IL-7R), but not CD25 (IL-2R) (Figure 3C

143 and S1D).

144

145 **$CD20^+$ T cells are IFN-γ producing T_H1/T_C1 cells in healthy volunteers and OC patients**

146 To further characterize the $CD20^+$ T cell population, intracellular cytokine stainings were performed to

147 identify T_H1/T_C1, T_H2/T_C2 or T_H17 cells. $CD3^+$ 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 $CD3^+$ T cells as well as the $CD20^-$ T-cell population

150   contained both IFN-γ (Figure 4A), IL-4 (Figure 4B) and IL-17 (Figure 4C) producing cells, with

151    $CD45RO^+$ memory T cells being largely responsible for cytokine secretion (Figure 4A-C; left panels).

152   However, within the $CD20^+$ T cell population, that consisted of >95% $CD45RO^+$ memory T-cells,

153   almost all cells produced IFN-γ, 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 $CD20^+$ T cells is increased in ascites fluid of ovarian cancer patients**

159   Patients with ovarian cancer had $CD20^+$ T cell populations in the peripheral blood that closely

160   matched that found in healthy volunteers, including a predominant T_H1/T_C1 T_{EM} phenotype (Figure

161   5A), with perhaps a minor trend towards a more T_{TD} phenotype (Figure 5D). However, in peritoneal

162   ascites fluid of OC patients the population of $CD20^+$ T cells was significantly expanded and comprised

163   approximately 23.4±6.8% of all ascites fluid T cells (Figure 5B; left panel and Figure 5C). These

164    $CD20^+$ ascites fluid T-cells were predominantly of T_{EM} phenotype (Figure 5B and D) and were also

165   skewed towards the T_H1/T_C1 cytokine production (Figure S3A-C).

166

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-γ.

171 Further, this CD20^{+} T-cell population was significantly enriched in ascites fluid in ovarian cancer

172 patients.

173 Data from the current study suggests that CD20^{+} 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 [13]. Concurrent with uptake of CD20 a similar uptake of HLA-DR by T cells

176 was detected. It is therefore conceivable that CD20^{+} 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^{+} T cells in cord blood seems to support a role for the development of

179 CD20^{+} 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±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^{+} CD20^{+} T cells in the peripheral blood of German and

185 British cohorts of healthy volunteers and patients with rheumatoid arthritis [19,20].

186 Importantly, as CD20 is considered a prototypical B cell marker, studies on CD20^{+} 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^{+} T cells in peripheral blood may be an artifact of flow cytometry

189 resulting from doublets.[21] However, we and others have demonstrated that single isolated T cells can

190 and do have CD20 molecules at their cell surface.[20] 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^{+} T cell phenotype by confocal microscopy did not

194 reveal any signs of B cell-T cell doublets, whereas we could clearly identify CD3^{+} CD20^{+} cells with

195 CD20 levels distinct from a B cell (~10-100 fold lower). Third, CD3^{+} CD20^{+} T cells could be isolated

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

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

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

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

226   cells.

227   In conclusion, we describe here the in depth characterization of CD20$^+$ T cells from peripheral blood

228   as CD8$^+$ effector memory and IFN-γ 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$^+$ 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

Materials & Methods

Antibodies and reagents

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

Cell lines and trogocytosis assays

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

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

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

Cell surface immunofluorescence staining

For determining the percentage of CD20^{+} T cells, 0.5×10^6 cells per indicated condition were stained with anti-CD3-CyQ, anti-CD19-PE and anti-CD20-FITC. For phenotypic characterization of T cells, 0.5×10^6 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   2Rα, IL-7Rα or HLA-DR was determined by staining 0.5×10^{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-IFNy, 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. Sjollema.

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_2O_2 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 ± 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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397

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$^-$ and CD20$^+$ 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$^+$ 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$^+$ 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$^+$ population. Asterisks represent significant

410 changes compared to PBMCs alone.

411

412 **Figure 2. *In situ* identification of CD3$^+$ CD20$^+$ 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 were individual CD3$^+$ CD20$^+$ T cells could be identified. **C** single cell

415 expression of CD3 and CD20 was validated using counterstaining with DAPI. **D** CD20$^-$ CD3$^+$ T-cells

416 and **E** CD20$^+$ CD3$^-$ B cells could also be readily identified.

417

418 **Figure 3. Phenotype of CD20$^+$ 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$^+$ T

427 cells. **D** CD20$^+$ 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$^+$ 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$^+$ CD20$^-$ and CD3$^+$ CD20$^+$ 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$^+$ 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$^+$ 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

Supplementary Figure legends

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

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

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