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Features of Human CD3⁺CD20⁺ T Cells

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Monoclonal Abs against CD20 reduce the number of relapses in multiple sclerosis (MS); commonly this effect is solely attributed to depletion of B cells. Recently, however, a subset of CD3⁺CD20⁺ T cells has been described that is also targeted by the anti-CD20 mAb rituximab. Because the existence of cells coexpressing CD3 and CD20 is controversial and features of this subpopulation are poorly understood, we studied this issue in detail. In this study, we confirm that 3–5% of circulating human T cells display CD20 on their surface and transcribe both CD3 and CD20. We report that these CD3⁺CD20⁺ T cells pervade thymus, bone marrow, and secondary lymphatic organs. They are found in the cerebrospinal fluid even in the absence of inflammation; in the cerebrospinal fluid of MS patients they occur at a frequency similar to B cells. Phenotypically, these T cells are enriched in CD8⁺ and CD45RO⁺ memory cells and in CCR7⁺ cells. Functionally, they show a higher frequency of IL-4⁺, IL-17⁺, IFN-γ⁺, and TNF-α⁺-producing cells compared with T cells lacking CD20. CD20-expressing T cells respond variably to immunomodulatory treatments given to MS patients: they are reduced by fingolimod, alemtuzumab, and dimethyl fumarate, whereas natalizumab disproportionally increases them in the blood. After depletion by rituximab, they show earlier and higher repopulation than CD20⁺ B cells. Taken together, human CD3⁺CD20⁺ T cells pervade lymphatic organs and the cerebrospinal fluid, have a strong ability to produce different cytokines, and respond to MS disease modifying drugs.

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Targeting CD20 with depleting mAbs is an approved therapy in rheumatic diseases (1) and is promising in multiple sclerosis (MS) (2–4). The therapeutic success has so far solely been attributed to the depletion of CD20⁺ B cells because CD20 is commonly considered as a specific B cell marker. In fact, CD20 is expressed during B cell development and maturation from pre-B cells to plasmablasts (5, 6). However, there are conflicting reports that CD20 is also expressed by some T cells. The first report of CD20-expressing T cells in the circulation dates back to 1993, where a subset of CD3⁺CD20⁺ T cells has been described in healthy subjects (7) and later also in peripheral T cell lymphoma (8). The description of CD3⁺CD20⁺ T cells in rheumatoid arthritis patients and controls (9) was disputed and has been regarded as an artifact of flow cytometry (10). Yet recently, a subset of CD3⁺CD20⁺ T cells that is also targeted by rituximab has been described (11). The issue remains complicated, however, because CD3⁺ B cells have been described as a result from ex vivo storage of blood samples, leading to contact-dependent Ag exchange between T and B lymphocytes (12).

We therefore aimed to clarify this issue by analysis of the expression of CD20 on human T cells in detail. First, we confirmed that indeed ~3–5% of human T cells in blood display CD20 on the surface and also transcribe both CD3 and CD20. We then examined their occurrence in human primary and secondary lymphatic organs and also in the cerebrospinal fluid. We determined their phenotype and their ability to produce cytokines. Furthermore, we analyzed how these cells are affected by different immunomodulatory treatments in MS patients. Thus, this study increases our knowledge about the biology of human CD3⁺CD20⁺ T cells and their response to immunomodulatory treatments in MS.

Materials and Methods

Patients and control donors

All human samples were collected following written informed consent according to local ethics policy guidelines of the Ludwig-Maximilian University and the German Heart Center in accordance with the Declaration of Helsinki. Peripheral blood was obtained from patients with a confirmed diagnosis of MS (n = 39); neuromyelitis optica spectrum disorder (NMOSD) (n = 18), who either were untreated or received several courses of rituximab, alemtuzumab, natalizumab, fingolimod, or dimethyl fumarate and healthy controls (HC, n = 11). Clinical data of MS and...
NMO/SD patients included in this study for therapy response are depicted in Supplemental Table I. Cerebrospinal fluid samples were obtained from a total of 14 untreated patients, 6 of which had MS (n = 6). Eight patients did not show any evidence of CNS inflammation and were classified as other neurological diseases (OND) (status epilepticus, n = 1; stroke, n = 3; cluster headache, n = 1; intracerebral bleeding, n = 1; and dementia, n = 2).

Bone marrow was obtained by iliac crest aspiration for diagnostic reasons. In addition, we received thymic tissue from five infantile patients (01, male, 87 d old; 02, female, 10 d old; 03, male, 93 d old; 04, male, 10 d; and 05, female, 7 d), which was removed during heart surgery.

Preparation of tissue samples from thymus, adenoids, bone marrow, blood, and cerebrospinal fluid. Thymic and lymphatic tissue were minced into very small fragments by gentle mechanical disruption and stirred in RPMI 1640 medium on ice (2 × 10 min) to obtain single-cell suspensions. PBMC were prepared by Pancoyl (Pan Biotech, Aidenbach, Germany) density gradient centrifugation. Bone marrow samples, anticoagulated with EDTA, were prepared by collagenase. Samples were incubated for 12 min in lysis buffer and further washed with PBS buffer, according to the manufacturer’s protocol (Beckman Coulter, Krefeld, Germany).

Flow cytometry

Single lymphocytes of human PBMC were identified by forward scatter of light (FSC) and side scatter of light (SSC). We further divided the lymphocytes into CD3+CD19- T cells, CD19+CD20- B cells and CD3+CD19+CD20+ T cells by FACSVerso (BD Biosciences, San Jose, CA) using APC-conjugated anti-CD3 (clone SK1), PE-conjugated anti-CD19 (clone MAb11; BD Biosciences), PerCPCy5.5–conjugated anti-CD20 (clone 2H7; eBioscience, San Diego, CA) Abs. Detailed phenotypic marker expression of each cell type was determined by surface staining in FACS buffer (PBS containing 1% BSA and 0.1% NaN3) with the following fluorochrome-labeled mAbs: FITC conjugated anti-CD3 (clone HIB19), APC-conjugated anti-CD8 (clone SK1), PE-conjugated anti-CD4 (clone OKT4), PerCP–Cy7–conjugated anti-CD20 (clone 2H7), PerCP-conjugated anti-CD20 (clone L27), APC-conjugated anti-CD3 (clone HIT3a), V450-conjugated anti-CD86 (clone FUN-1), FITC conjugated anti-HLA-DR (clone L423), efluor450 Fluor–conjugated anti-CD40 (clone SC53), PE–conjugated anti-CD45 RA (clone H100), PE-conjugated anti-CD45 RO (clone UCHL1), FITC-conjugated anti-CD27 (clone LG.7F9), PE–conjugated anti-CD19 (clone SJ25C1), efluor450–conjugated anti-CD38 (clone HIT2), and APC-conjugated anti-CCR7 (clone 3D12). All Abs were purchased from eBioscience or BD Biosciences. The CD20 cutoff for negative versus positive expression of each cell type was determined by surface staining in FACS buffer and further determined the CD20 expression (Supplemental Fig. 1B). The display of CD20 on a subset of T cells is not due to doubltots between B and T cells, because these cells display a lower level of CD20 than B cells and lack markers typically found on B cells such as HLA-DR, CD40 (Fig. 1), and CD19.

We compared the phenotype of CD3+CD20+ T cells and CD3+CD20- T cells. Fig. 1 shows one representative sample and the statistical evaluation of measurements from six different healthy donors. CD3+CD20+ T cells contained a lower percentage of CD4+ cells, correspondingly more CD8+ cells (p < 0.0001; Fig. 1A, 1B, 1K, less CCR7+ cells (p < 0.001; Fig. 1C, 1K), and more CD45RO+ cells (p < 0.001; Fig. 1D, 1K) than CD20- T cells. HLA-DR, an activation marker for human T cells, was hardly observed on CD3+CD20+ T cells (Fig. 1E). CD3+CD20+ and CD3+CD20- T cells expressed CD27+ and CD86+ similarly (Fig. 1F, 1G). All lymphocyte subsets including CD3+CD20+ T cells displayed CD49d (VLA-4), the target of natalizumab, on the surface (Fig. 1H). CD20+ T cells transcribe both CD3 and CD20 We sorted CD3+CD20- cells, CD3+CD20- cells, and CD3+CD20+ B cells from healthy donors to compare their transcript levels of CD3, CD19, and CD20 by quantitative PCR. Thereby we confirmed that CD20+ but not CD20- T cells transcribe CD20 (p = 0.0076) (Fig. 2). Accordingly, CD3+CD20- T cells transcribed only CD3 but not CD19 or CD20. As expected, CD19+CD20- B cells transcribed CD19 and CD20 but not CD3 (Fig. 2). B cells expressed ~15-fold higher levels of CD20 transcripts than CD3+CD20- T cells reflecting the greater surface expression of CD20 on B cells (Fig. 2, Supplemental Fig. 1B). Cytokine production by CD20+ T cells We analyzed the ability of CD20 expressing T cells from healthy donors to produce cytokines upon stimulation with PMA and ionomycin. Thereby we found that CD3+CD20+ T cells produce more readily IL-4, IL-17, TNF-α, and IFN-γ than CD3+CD20- T cells (Fig. 3, Supplemental Fig. 1C). Furthermore, we analyzed the cytokine production of CD4+ or
CD8+ and CD45RA+ or CD45RO+ subsets of CD3+CD20+ and CD3+CD20− T cells. This showed that CD4+CD3+CD20+ T cells produced more IL-4, IL-17, and TNF-α than CD8+ CD3+CD20+ T cells, whereas CD45RO+CD3+CD20+ T cells produced more TNF-α than CD45RA+CD3+CD20+ T cells (Supplemental Fig. 2). In comparison with CD19+CD20+ B cells, each analyzed cytokine was expressed by a greater proportion of CD3+CD20− T cells.
Using clone 2H7, we found that 3.8 ± 0.6% (mean ± SEM of 11 independent experiments from 11 different donors) of all lymphocytes in human blood from healthy donors display CD20 (Fig. 4). We detected CD20-expressing T cells not only in blood (Fig. 4) but also in adenoids (3.9 ± 0.45%) and in the bone marrow at a frequency of 8.0 ± 0.32% (Fig. 4). In addition, we studied the presence of CD3+CD20+ T cells in the thymus of young children who underwent cardiac surgery. We found CD3+CD20+ in three of five thymi in mature medullary thymocytes (CD4+CD8− or CD4−CD8+) (Fig. 4).

CD3+CD20+ T cells in the cerebrospinal fluid in the absence and presence of CNS inflammation

We examined the presence of CD3+CD20+ T cells in the cerebrospinal fluid of donors with and without signs of inflammation in the CNS. We analyzed cerebrospinal fluid samples from eight OND patients, who did not show pleocytosis, a disturbance of the blood–brain barrier, or intrathecal IgG production. There we detected CD3+CD20+ T cells regularly, namely with a mean frequency of 4.7 ± 1.2% (Fig. 4). In the cerebrospinal fluid of MS patients at relapse, we observed CD3+CD20+ T cells with a similar frequency of 2.9 ± 1.3% (mean ± SEM of six patients) of
significant difference in the frequency of CD3+CD20+ T cells in blood or CSF donors. In MS patients, CD3+CD20+ T cells and CD19+CD20+ B cells occurred at a similar frequency (2.9 ± 3.2% and 2.6 ± 0.3%, respectively; mean ± SEM of six patients).

**CD20-expressing T cells respond to immunomodulatory MS therapies**

Although we noted a similar frequency of CD3+CD20+ T cells in the blood of untreated MS patients, OND patients and healthy controls (Fig. 4), we observed a strong response of this T cell population to disease modifying treatments (Fig. 5). We analyzed the effect of natalizumab, alemtuzumab, dimethyl fumarate and fingolimod on blood cells of MS patients in a cross-sectional study in comparison with untreated MS patients. We analyzed both the absolute number (Fig. 5A) and the percentage of CD3+CD20+ T cells compared with all lymphocytes (Fig. 5B). Natalizumab increased the absolute number of CD20 expressing T cells, while alemtuzumab, fingolimod, and dimethyl fumarate decreased it (Fig. 5A). In addition, natalizumab also increased the relative frequency of CD3+CD20+ T cells compared with all lymphocytes (Fig. 5B).

Rituximab depleted CD3+CD20+ T cells in peripheral blood (Fig. 6). We went on to compare the recovery of CD3+CD20+ T cells in comparison with CD19+CD20+ B cells after rituximab treatment. For this part of the study, we used both MS and NMOSD patients. CD20-expressing T cells replenished earlier and in higher frequencies than B cells. Although in untreated patients CD20+ B cells were more frequent in blood than CD20+ T cells, this ratio inverted in the recovery phase (Fig. 6). Detailed replenishment kinetics of 8 patients revealed an earlier appearance and higher frequency of CD20+ T cells in comparison with CD19+ CD20+ B cells following a course of rituximab (Supplemental Fig. 3). Of these eight patients (Supplemental Fig. 3B, 3D, 3E), 3 relapsed 4–8 mo after the last rituximab infusion, at a time when there were more CD3+CD20+ T cells than B cells in the blood. In one of these patients (Supplemental Fig. 3D), a relapse occurred when CD20-expressing T cells had reached 4% of all lymphocytes, whereas CD20+ B cells were hardly detectable in blood.

**Discussion**

This study shows that human CD20-expressing T cells pervade primary and secondary lymphatic tissues, blood, and cerebrospinal fluid. CD3+CD20+ T cells produce more readily the cytokines IL-4, IL-17, TNF-α, and IFN-γ than CD3+CD20- T cells and respond to immunomodulatory drugs.

Our analysis demonstrates a CD3+CD20+ population comprising 3–5% of all lymphocytes in human blood, corroborating previous reports (11, 13). This cell population is a T cell subset because these cells transcribe CD3 at a similar level as conventional T cells and lack surface molecules found on B cells such as CD19, CD40, and HLA-DR. All this justifies to call this population CD20 expressing T cells. This CD20-expressing T cell subset is clearly distinct from a recently described population of CD3-expressing B cells that were reported to be an artifact based on storing conditions (12) for the following reasons: first, the CD20-expressing T cells described in this article display T cell markers, not markers typically found on B cells such as CD19, CD40, and HLA-DR; second, they transcribe both CD3 and CD20; and third, this population is seen in direct ex vivo analyses without prior storage.

An intercellular transfer of cell surface proteins from APCs to T cells may follow TCR triggering, a process called trogocytosis (14). In vitro, T cells may acquire CD20 from B cells within minutes (15). Thus, in theory, the CD3+CD20+ T cells present in human blood could display CD20 because of trogocytosis or because these T cells transcribe it themselves. To address this issue, we have sorted CD3+CD20+ T cells directly from blood and determined the transcription of CD20. We found that—in contrast to conventional T cells—this T cell subset transcribes CD20, consistent with recent data (11). We also found that these CD3+CD20+ T cells do not display HLA-DR, which is commonly transferred to T cells during trogocytosis (16). Also, other molecules typically found on B cells such as CD19, HLA-DR, and CD40 were absent on CD3+CD20+ T cells. The presence of CD20 transcripts together with the absence of HLA-DR implies that CD20+ T cells present in human blood produce CD20 themselves.

The transcript level of CD20 was lower in CD3+CD20+ T cells than in B cells, further reflected by the lower level of surface expression of CD20 in this T cell subset as compared with B cells. The lower surface expression of CD20 in CD3+CD20+ T cells compared with B cells explains why detection of CD20 on this T cell subset can only be achieved with a high-affinity anti-CD20 mAb (such as the mAb 2H7 as we show in this article). This may partly explain conflicting reports about the existence of CD3+CD20+ T cells.

Our phenotypic analysis showed that CD3+CD20+ are enriched in CD8+ T cells and cells expressing the memory marker CD45RO. They show a higher percentage of T cells that lacked the chemokine receptor CCR7. Although CCR7+ T cells include central memory T cells in lymphatic tissue, T cells lacking CCR7...
include effector memory T cells, which are enriched in peripheral organs and show a high propensity to produce IFN-γ (17). In agreement with this, we found that CD20+ T cells contain after stimulation a higher proportion of IL-4+, IL-17-, IFN-γ-, and TNF-α–producing cells than conventional CD3+CD20+ T cells, whereas previously production of IL-17 by this subpopulation in rheumatoid arthritis has been reported (13). Taken together, CD3+CD20+ cells are a heterogeneous group of CD20 transcribing T cells comprising ∼3–5% of human blood lymphocytes and are enriched in CD8+ T cells with phenotypic and functional features of effector memory T cells.

We identified that these CD20+ T cells pervade human primary (thymus and bone marrow) and secondary (adenoid) lymphatic tissue. Within the thymus we detected this T cell population within the mature, CD4 or CD8 single positive medullary T cell population. This might indicate that these cells originate from the thymus, although also evidence for a migration from mature T cells back to the thymic medulla has been reported (18, 19).

Further, our work reveals that CD20 expressing T cells are present in the cerebrospinal fluid in the absence of inflammation. For immunosurveillance of the brain, immune cell entry into the cerebrospinal fluid–drained spaces is an actively regulated process. Thereby, the immune cell composition in the cerebrospinal fluid can be highly selective as opposed to merely reflect the immune cell population in the blood (20).

In MS and other neuroinflammatory diseases, we observed these T cells in the cerebrospinal fluid, albeit not enriched compared with blood. We noted that these cells are in the cerebrospinal fluid in MS about as abundant as CD20-expressing B cells. The presence of such cells was also reported within tissue lesions of MS patients (21), which could also indicate their participation in this disease.

We found that these CD20+ T cells are enriched in CD8+ cells. CD8+ T cells are more abundant in MS lesions than CD4+ T cells in MS lesions (22, 23). The role of CD8+ T cells is expected to include both cytotoxic (24) and regulatory features (25).

We went on to analyze the response of these CD20-expressing T cells to different immunomodulatory treatments in MS. The number of approved drugs to treat MS has increased enormously in recent years: these show different modes of action, some of which are only partly understood (26). CD20-expressing T cells in blood were depleted by rituximab, in accordance with others (11). The effect of rituximab on B cells and T cells in the cerebrospinal fluid has also been examined: rituximab largely depletes B cells in the cerebrospinal fluid, although not in all patients as complete as in blood (27, 28). Interestingly, rituximab reduces also the number of T cells in the cerebrospinal fluid to about half of the original number (27). We found that ∼5% of all T cells in the cerebrospinal fluid express CD20. Thus, the depletion of T cells in the cerebrospinal fluid is presumably because of two effects: elimination of CD3+CD20+ T cells and reduction of CD3+CD20+ T cells because of a diminished inflammation.

The timing of a maintenance therapy during rituximab treatment is a clinically relevant issue. The reappearance of CD20-expressing B cells was previously linked to new relapses in NMO patients (29). B cell subsets reconstitute different after rituximab, initially only naive B cells with a delayed appearance of memory B cells; the reappearance of memory B cells may suggest that a maintenance therapy with rituximab should be performed (30). We ascertained that the replenishment of CD20-expressing T cells occurs much earlier than that of CD20-expressing B cells. In fact, 3 mo after rituximab, CD20-expressing T cells were more frequent in blood than CD20-expressing B cells. We observed that one patient un-
derwent a relapse when CD3$^+$CD20$^+$ T cells were replenished to normal levels, but B cells were still depleted. Thus, future studies will clarify whether monitoring of the earlier reappearance of CD20-expressing T cells results in optimization of anti-CD20 therapies.

Dimethyl fumarate, which has a differential effect on circulating lymphocytes (31) and fingolimod, which retains mainly CCR7$^+$ T cells in the lymph node (32) reduced CD20$^+$ T cells in the peripheral blood. We found that natalizumab, which blocks α4 integrin–mediated adhesion (33), has a disproportionally strong effect on CD3$^+$CD20$^+$ T cells indicating that the migratory pattern of CD20-expressing T cells is highly regulated by α4 integrin.

In summary, the intrathecal presence of CD20-expressing T cells, their strong propensity to cytokine production and their response to disease modifying drugs suggest a role in the pathogenesis of MS. Effects on CD20-expressing T cells should be considered when elaborating mechanisms of action of different immunomodulatory drugs and monitoring of CD20-expressing T cells might help to optimize therapy in MS and NMOSD.

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Disclosures

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