TNFR2 expression is a hallmark of human memory B cells with suppressive function

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Tumor Necrosis Factor Receptor 2 (TNFR2) expression is increasingly being linked to tolerogenic immune reactions and cells with suppressor function including a subset of T-regulatory cells. B-regulatory cells play an important role in control of T-cell responses and inflammation. Recently, we described TNFR2 as a marker for IL-10-producing B cells, a hallmark of this cell subset. Here, we demonstrate that proliferation of T cells is reduced in the presence of TNFR2 positive human memory B cells generated with TLR9 ligand, while TNFR2- and TNFR2+CD27- B cells display costimulatory activity. Our data further reveal that IL-10 secretion is characteristic of IgM+ naïve and memory B cells but suppressive activity is not restricted to IL-10: (i) the inhibitory effect of TNFR2+ switched memory B cells was comparable to that exerted by TNFR2+ IgM+ memory B cells although IL-10 secretion levels in the cocultures were lower; (ii) supernatants from TNFR2+ memory B cells failed to suppress T-cell proliferation. Based on our findings, we propose that formation of Breg is a specific characteristic of human memory B cells undergoing terminal differentiation. Our data further corroborate that TNFR2 represents a viable marker for identification of memory B cells with regulatory function.

Keywords: B cells · Breg · IL-10 · TLR9 · TNFR2

Introduction

Next to forming a reservoir for immune memory and antibody production, B cells play an important regulatory role, which is mainly based on release of immunosuppressive cytokines. In recent years, there has been increasing evidence that the B cell-derived human lymphotoxin (Lta1B2) is essential for remodeling processes in secondary and tertiary lymphoid organs, while other cytokines (e.g. TNF, IFN-γ, GM-CSF, IL-6, IL-17, IL-2) released by B cells shape effector and memory CD4+ T-cell responses. Additionally, B cells secrete cytokines (e.g. IL-10, IL-35) that counteract inflammatory responses and act as potent regulators in allergy and autoimmune disease, cancer, infection, and transplant rejection [1–3].

The main B-lymphocyte subpopulations in human peripheral blood are naïve CD27 negative (CD27-), which are considered the first line of defense against pathogens and produce low-affinity IgM. They comprise IgM+ CD21+ follicular (FO) B cells II, along

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with FOI and transitional B cells [4]. Among this fraction, transitional B cells (IgM+ CD38hi CD27-) are most prominently reacting to TLR9 stimulation [5]. Memory B cells (CD27+) can be divided into switched memory B cells (IgM-CD27+) and the IgM memory B cells (IgM+CD27+). While the former subpopulations have undergone class switch recombination, the latter share some of the characteristics including affinity maturation. IgM+ memory B cells are strongly responsive to CpG stimulation [6]. They show higher similarity in their gene expression profiles with classical IgG+ memory B cells than with naïve B cells [7].

To date, it is well accepted that production of the anti-inflammatory cytokine IL-10 is the hallmark of B cells with regulatory function. IL-10 can be produced by many different B-cell subsets depending on stimulation, organ, and species [8, 9]. However, characteristic surface markers that allow phenotypical identification of these cells have, so far, not described [9, 10]. Interestingly, an important role of B-regulatory cells characterized by IL-10 production is demonstrated with a fact, that in many immunological disorders the percentage of these cells differ from healthy controls. While in some autoimmune diseases (e.g. MS, systemic lupus, rheumatoid arthritis) these cells are expanded, in other (e.g. myasthenia gravis, neumyelitis optica) these cells are underrepresented compare to healthy controls [1–3, 11–13]. Notably, in murine models and cancer patients, B-regulatory cells promote tumor progression by interfering with antitumor immunity via suppression of the T cell-mediated immune response [14].

Tumor necrosis factor is a central regulator of inflammation, which exerts a variety of functions during an immune response and acts in two distinct forms — membrane bound (mTNF) or soluble (sTNF). These variants activate two different receptors – TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), characterized by distinct affinity to TNF and differential signaling pathways that lead to divergent immune effector functions. While TNFR1 is constitutively expressed on nearly all nucleated cells and can be activated by both forms of TNF, TNFR2 is only present on specific cell subpopulations and only activated by mTNF. Notably, mTNF is a precursor of soluble tumor necrosis factor, which is released by the TNFα converting enzyme [15–20]. Moreover, the binding of mTNF to TNFR2 induces both intracellular signal transduction downstream to TNFR2 as well as reverse, inside-out signaling in the cells presenting the mTNF. Signaling mediated via mTNF is further bifunctional, for example, promoting increased TNF production and, at the same time, a negative regulatory response via secretion of TGF-β, which promotes resistance to LPS-induced inflammation in macrophages [21–25]. Albeit the function of TNFR2 was extensively studied on DCs, monocytes, and T cells [22, 26–29], and there is evidence that soluble TNFR2 could be used as a biomarker in a variety of inflammatory disease states [30–32], the role of this receptor on human B cells remains to be investigated. As demonstrated earlier by our group, TNFR2 expression is inducible on a variety of peripheral B-cell subpopulations and its presence coincides with TLR9-triggered formation of IL-10 and antibody-secreting B cells [33]. Here, we provide novel evidence that TNFR2 characterizes B cells' regulatory potential.

Results

Targeted stimulation of IL-10 release in B cells reduces T-cell proliferation

Previous reports demonstrate that B cell-derived IL-10 suppresses T-cell proliferation and thereby contributes to control of immune responses [34, 35]. Here, we confirmed previous findings by demonstrating the downregulation of T-cell proliferation in PBMC stimulated with anti-CD3/28 beads in the presence and absence of recombinant IL-10. Decreased proliferation in the presence of IL-10 (quantified by CFSE dilution) was detectable in both CD4+ and CD8+ T-cell subsets (Fig. 1A).

Next, we assessed whether the presence of B cells secreting IL-10 interferes with T-cell proliferation. PBMC depleted of CD19 B cells were supplemented with B cells pretreated for 2 days with either CpG 2006 ODN or UV-irradiated BHK cells with and without CD40L expression or combinations thereof because stimulation of human B cells with CpG leads to production of IL-10 and combination with CD40L acts synergistically [6, 34, 35]. Indeed, CD4+ and CD8+ T-cell proliferation was reduced in many donors when B cells added were prestimulated with CpG and particularly in combination with CD40L (Fig. 1B). Subsequent analysis of cell-free supernatants confirmed that IL-10 levels originated from cultures containing B cells stimulated with CpG and CD40L had the highest levels of IL-10 (Fig. 1C).

TNFR2-expressing B cells are present in healthy donors and CVID patients

In a previous study, we demonstrated that stimulation of human peripheral B cells with TLR9 ligand CpG triggers an upregulation of TNFR2 expression and coincides with IL-10 production, and could, thus, be used for enrichment of IL-10 producing B cells [33]. Here, we confirm the presence of TNFR2+ B cells in peripheral blood of healthy donors and Common Variable Immunodeficiency (CVID) patients (Fig. 2A). Similar to our earlier observations, this B-cell subset is increased upon stimulation with CpG ODN 2006. However, the reduced frequency of this B-cell subpopulation in CVID patients coincided well with decreased numbers of IL-10 secreting B cells described in this patient population [36, 37].

TNFR2 expression characterizes B cells that negatively control T-cell proliferation

To study the effect of TNFR2-expressing B cells on T-cell proliferation, we stimulated B cells with CpG ODN 2006 and sorted B-cell fraction with and without TNFR2 expression (Fig. 2D). The results from seven independent donors demonstrated that sorted TNFR2 positive B cells reduced proliferation of CD4+ T cells after polyclonal stimulation with anti-CD3/CD28 when compared to...
TNFR2 negative B cells (Fig. 2B). In line with our previous findings, supernatants of cocultures supplemented with TNFR2 negative B cells displayed lower IL-10 levels when compared to those containing TNFR2 positive B cells (Fig. 2C). These results confirm that in our experimental setting, TNFR2-expressing B cells represent B cells with regulatory function.

TNFR2+ subset of TLR9-stimulated B cells differs in IL-10 production and suppressive capacity

Since B-cell subpopulations differ in their ability to secrete IL-10, we studied the suppressive effect of the three different subpopulations expressing TNFR2 upon CpG ODN stimulation (Fig. 3A). The presence of TNFR2 negative and TNFR2 positive naïve B cells (CD27-) increased the proliferation of CD4+ or CD8+ T cells when compared to PBMC depleted of B cells. On the contrary, TNFR2 positive memory B cells — both switched memory (CD27+IgM-) and IgM memory (CD27+IgM+) — decreased T-cell proliferation when compared to TNFR2 negative and TNFR2 positive naïve B cells fractions. Notably, the regulatory effect was more pronounced in CD4+ T cells, but was also observed in CD8+ T cells (Fig. 3B). Furthermore, IgM memory B cells induced stronger reduction of CD4+ T-cell proliferation (more than 40% in 3/6 donors; in average 26% when compared to TNFR2 negative B cells) than switched memory B cells (with maximal reduction of 28% and average 19%).

This difference in suppressive capacity prompted us to ask whether the two TNFR2+ B-cell subsets differ in their suppressory mechanism of action. Analysis of IL-10 concentrations in
Figure 2. TNFR2 expression on B cells in CVID patients and influence of TNFR2+ and TNFR2- B-cell subpopulations on T-cell proliferation. (A) TNFR2 expression on B cells in PBMC of healthy donors (HD; n = 11) and CVID patients (CVID; n = 22) on unstimulated and CpG-stimulated B cells 3 days after stimulation. Results from n = 6 independent experiments are shown. Data were analyzed using unpaired t-test (*p = 0.0201). (B) Coculture of unstimulated and anti-CD3/28 stimulated PBMC without B cells (PBMC/ΔB) with B cells prestimulated for 2 days with CpG and sorted into TNFR2 positive and negative subpopulation (ratio of 2:1 B cells: CFSE-stained PBMC without B cells). Proliferation of CD4+ and CD8+ T cells was followed as dilution of CFSE dye on day 4 (n = 7 independent donors from three independent experiments) (left panel). Histograms of CFSE dilution in CD4+ T cells from one representative of seven donors analyzed (left panel) and summary of percentages of CFSElow CD4+ and CD8+ T cells (right panel). Data were analyzed using two-tailed Wilcoxon matched-pairs signed rank test (*p = 0.0156). (A-B) All data were measured by flow cytometry. The bars show the means (±SD) of the percentages of proliferating (CFSElow) T cells. Results of individual donors are depicted in different colors. (C) IL-10 production measured in supernatants from the cocultures of unstimulated or stimulated PBMCs with B cells on day 4 determined by ELISA (n = 7 independent donors from 3 independent experiments). The bars show the means (±SD). Data were analyzed using two-tailed Wilcoxon matched-pairs signed rank test (*p = 0.0156). (D) Flow cytometry gating and sorting strategy for TNFR2 positive and negative B cells.

coculture supernatants revealed that the cocultures of stimulated PBMC with switched memory B cells contained only low concentrations of IL-10 when compared to the other conditions including cocultures with TNFR2 negative B cells (Fig. 3C).

Since the use of whole PBMC in our experiments is relevant because it reflects the in vivo situation more closely, it has the disadvantage that the contribution of single-cell subpopulations is hard to discern. To understand and exclude an effect of other cell types, we performed the same experiment with isolated CD4+ T cells. The results (Fig. 4A) confirmed the effects observed in the PBMC cocultures: reduced proliferation of T cells was found in the presence of IgM memory and switched memory B cells when compared to TNFR2 positive CD27 negative or TNFR2 negative B cells. Analysis of IL-10 in the coculture supernatants (Fig. 4B) showed lower levels in all conditions, but, the distribution pattern resembled that in cocultures with PBMC. Again, high concentrations of IL-10 were detected in the supernatants of TNFR2 positive CD27 negative and IgM memory B cells.

TLR9-stimulated B cell-derived soluble factors do not suffice for suppressing T-cell proliferation

Since IL-10 has been viewed as the mainstay of suppressive B-cell function and the major other mechanisms described are also attributed to cytokine mediators, we sought to confirm that soluble factors are responsible for the regulatory effects of TNFR2 positive B cells. To address this question, supernatants of the sorted
Figure 3. Influence of different B cells subpopulations on T-cell proliferation. (A) Gating and sorting strategy for TNFR2- and TNFR2+CD27- (T2+CD27-), TNFR2+ switched memory (T2+IgM-CD27+), and TNFR2+ IgM memory (T2+IgM+CD27+) B cells. (B) Coculture of anti-CD3/28 stimulated PBMC without B cells (PBMCΔB) (ratio of 2:1 B cells: CFSE-stained PBMCΔB) with addition of IL-10 or different B-cell fractions: B cells were pres- tumulated for 2 days with CpG ODN 2006 and then sorted into four B-cell subpopulations: TNFR2-, T2+CD27-, T2+IgM-CD27+ and T2+IgM+CD27+. Histograms showing CFSE dilution of dividing CD4+ T cells on day 4 from one representative of six donors (upper panel) and summary of percentages of CFSElow CD4+ and CD8+ T cells (lower panel). Proliferation of CD4+ and CD8+ T cells was quantified via CFSE dilution on day 4 (n = 7 independent donors from four independent experiments) (upper panel). Representative histograms from one representative donor of seven are presented in the lower panel. Blue background marks the level of T-cell proliferation in the condition containing TNFR2+CD27+IgM+B cells. Data were analyzed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey’s correction for multiple comparisons tests. (A–B) All data were measured by flow cytometry. (C) IL-10 production was measured in supernatants collected from day 4 PBMC/B-cell cocultures by ELISA (n = 7 independent donors from four independent experiments). Data were analyzed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey’s correction for multiple comparisons tests. Bars provide the mean values (±SD). Results of individual donors are depicted with different colors.

B-cells fractions (TNFR2-, TNFR2+CD27-, TNFR2+CD27+IgM, TNFR2+CD27+IgM+) were collected after 2 days of B-cell stimulation with CpG ODN 2006 and added to PBMC, which were subsequently stimulated with anti-CD3/CD28 microbeads. Although IL-10 was detectable in all supernatants, no reduction in proliferation of CD4+ or CD8+ T cells was observed in PBMC treated with the respective supernatants (Fig. 5). These data indicated that suppressive B-cell function might require cell-cell contact additionally to IL-10 secretion.

Discussion

TNFR2 expression has been associated with suppressive and tolerogenic roles in the past and characterizes a subset of CD4+CD25+ Treg cells with high levels of FoxP3 and suppressive activity [38]. The TNF-TNFR2 interaction was further shown to contribute to suppressive function and to promote Treg activation, expansion and survival [27, 39, 40]. We previously described that in human B cells TNFR2 expression coincides with IL-10 production [33]. In this study, we investigated the regulatory capacity of TNFR2+ B cells obtained via stimulation with TLR9 ligand CpG ODN 2006.

Our results confirm earlier reports that TLR9-stimulated human B cells secrete IL-10 [6, 34, 35] and that IL-10 promotes downregulation of polyclonal T-cell proliferation (Fig. 1A). In contrast to previously published data, in the PBMC setting used in this study, the reduction of T-cell proliferation observed after enrichment of PBMC with CpG-stimulated B cells was not statistically significant, but significance was obtained upon...
addition of CD40L for B-cell stimulation [34, 35] (Fig. 1B). However, since our study focused on the role of TLR9-dependent, T cell-independent induction of B-regulatory cells, addition of CD40L, a surrogate for T-cell help, would have diverted the scope and was therefore abandoned in the following experiments.

Notably, in initial experiments suppression levels correlated well with IL-10 levels detected in the supernatants of the PBMC/B cell cocultures (Fig. 1C). However, the data obtained with purified B-cell fractions suggested that suppressive activity is not limited to IL-10, but rather involves additional secreted factors and possibly T-B cell contact (Fig. 4). Notably, in this study suppressive activity was observed as a relative phenomenon, because it could only be observed in relation to the costimulatory comparator, for example, TNFR2+ B cells or naive TNFR2+ B cells.

Figure 4. Coculture of different TNFR2+ B cells subpopulations with isolated CD4 positive T cells. (A) B cells were prestimulated for 2 days with CpG ODN 2006 and then sorted into four B-cell subpopulations: TNFR2−, T2+CD27−, T2+IgM−CD27+, and T2+IgM+CD27+.

These B subpopulations were cultured with CD4+ T cells (ratio of 2:1 B cells: CD4+ T cells) stimulated with anti-CD3/28 beads. Proliferation of CD4+ T cells was analyzed by CFSE dilution on day 4 by flow cytometry (n = 6 independent donors from two independent experiments). The blue background marks the level of T-cell proliferation in the condition with TNFR2+CD27+ IgM− B cells. Data were analyzed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey’s correction for multiple comparisons tests. (B) IL-10 production was determined by ELISA and measured in supernatants from co-cultures of stimulated CD4 positive T cells with B cells on day 4 by ELISA (n = 6 independent donors from 2 independent experiments). Data were analyzed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey’s correction for multiple comparisons tests. The bars represent the mean values (±SD). Results of individual donors are depicted with different colors.

Figure 5. Influence of supernatants from B-cells subpopulations on T-cells proliferation. Proliferation of gated (A) CD4+ and (B) CD8+ T cells was quantified by CFSE dilution in PBMC without B cells (PBMCΔB) treated with B-cell supernatants after 4 days (n = 5 independent donors from three independent experiments). Supernatants were generated after sorting and stimulation of the respective B-cell subpopulations (TNFR2−, T2+CD27−, T2+IgM−CD27+, and T2+IgM+CD27+) from four independent donors in three independent experiments, pooled for experiments and added to PBMC prior to anti-CD3/28 stimulation of T cells. Blue background marks the level of T-cell proliferation in the condition without supernatant supplementation. Data were analyzed using Friedman test (for CD4+: χ² = 6.52, for CD8+: χ² = 3.72). (A-B) All data were measured by flow cytometry. (C) IL-10 levels in the supernatants of the respective B-cell subpopulations used for these experiments were measured 4 days after sorting by ELISA (n = 4 independent donors three independent experiments). Bars provide the means (±SD). Results of individual donors are depicted with different colors.
In the human, different subsets of Breg have been described. While CD24hi CD38hi CD5+ B cells originate from transitional B cells [41–43], memory-derived (CD27+) B cells with regulatory function have been described to express TIM-1 [44], CD1d [45], or high levels of CD24 [46]; additionally, an IgG2a+ CD25+CD71+CD73+ B-cell population specific for bee venom allergen phospholipase A2 was described in nonallergic bee keepers [47]. Here, we investigated the functional impact of TNFR2-expressing human B cells on T-cell expansion. The results confirmed that the presence of TNFR2 on the B cell is associated with a regulatory function, for example, decreased proliferation in CD4+ as well as CD8+ T cells stimulated with anti-CD3/28 (Fig. 2B). Although recent reports indicated that CpG-triggered peripheral blood B-cell subsets released variable levels of IL-10, IL-6, and proinflammatory cytokines (i.e. TNF) with no evident subpopulation-specific pattern or marker for IL-10 [48, 49], higher IL-10 was detected in the supernatants containing TNFR2 positive B cells (Fig. 2C), well in accordance with our earlier findings [33]. These results indicated that the presence of IL-10 might be responsible for the suppressive effects as previously postulated [34, 35, 49]. Furthermore, these results are the first to demonstrate that TNFR2 expression is a specific marker for identification, characterization, and/or purification of B cells with regulatory function (Fig. 2B).

Memory B cells were previously reported to constitutively express TLR9 [50–52]. Earlier work from our group further demonstrated that IgM memory B cells (CD27+IgM+) are the major targets for CpG ODN and the main source of IL-10 after TLR9 stimulation and initiation of terminal differentiation to antibody-secreting cells [6, 33]. We, therefore, asked whether suppressive activity of TNFR2 positive B cells originates from this B-cell subset. To this end, we compared the suppressive capacity of TNFR2 negative B cells and three different TNFR2 positive subpopulations, for example, naïve (CD27−), switched memory (CD27+IgM−), and IgM memory (CD27+IgM+). As expected from our previous findings, CD4+ and CD8+ T-cell proliferation was downregulated in the presence of IgM memory cells, which was well in line with coinciding high levels of IL-10 in the supernatants (Fig. 3B and C). However, PBMC enriched with prestimulated switched memory B cells (CD27+IgM−) similarly decreased CD4+ and CD8+ proliferation despite displaying only low levels of IL-10 in supernatants. Comparable results were obtained when both TNFR2+ memory B cells subpopulations were cocultured with purified CD4+ and CD8+ T cells (Fig. 4). These data highlight that memory B cells are predisposed to exert suppressive function. Of note, reactivity of memory B cells to TLR9 ligand CpG might have introduced a bias by favoring the differentiation of memory B cells to Breg in our experimental setup. Nevertheless, memory B cells are equipped to differentiate more rapidly once activated and in the context of an immune response, thus, the formation of Breg could serve as an important control mechanism that dampens antigen presentation and prevents de novo priming and expansion of T cells in response to cognate antigen and controls Th differentiation. It may, thus, serve to avoid unnecessary energy consumption that instead is rerouted to the execution of the secondary immune effector response (Fig. 6).

Moreover, high levels of IL-10 were also detected in the supernatants with naïve CD27-TNFR2+ B cells (T2+CD27−), which did not affect T-cell proliferation (Fig. 3B and C). Of note, IL-10 production in CpG-stimulated naïve B cells — which in our case reached comparable levels to those in memory B cells — was also observed by other groups [44, 53]. These findings provided the notion that IL-10 production might represent a characteristic of IgM+ B cells (e.g. naïve, transitional (CD27−CD24++CD38+++), and IgM+ memory B cells). By contrast, according to our data, IL-10 might be less relevant for suppressive activity of switched memory B cells. This prompted us to question the solitary role of IL-10 in mediating T-cell suppression by B cells with regulatory function. Indeed, lack of suppressive activity in supernatants from TNFR2+ memory B cells (Fig. 5A and B) confirmed that cell-cell contact may be additionally needed to promote the suppressive effect. Interestingly, Bouaziz et al. showed that blocking of IL-10 only partially reversed the inhibitory effect of IL-10 producing B cells on T-cell proliferation [34]. Notably, in a recent report TGFβ secretion was found to arise from TIM-1+ CD27+ B cells, which was not observed in the CD27- fraction [44]. However, the authors of this study did not distinguish IgM+ from class-switched memory B cells. In another study on memory B cells, next to IL-10, PD-L1 expression was shown to contribute to suppression of T-cell proliferation and reduction of IFN-γ and IL-17 in stimulated CD4+ T cells [49]. There further is increasing evidence for IL-10-independent mechanisms accounting for regulatory function of B cells from autoimmune and inflammatory diseases. Interaction with adjacent cells via costimulatory surface receptors (PD-L1, ICAM-1, CD73, Fasl, CD80, CD86, CTLA-4) and secretion of suppressory mediators, such as IL-35, TGF-β, or granzyme B, have been described [14, 42, 54–56]. Moreover, a variety of effects beyond IL-10 secretion were suggested, for example, regulation of Treg function and expansion, induction of apoptosis in effector T cells, dampening of antigen presentation, and a negative impact on iNKT homeostasis [14, 42, 54, 56].

To further assess the relevance of soluble mediators in promoting the suppressory function of different B-cell subpopulations (TNFR2 negative, TNFR2 positive - naïve (T2+CD27−), switched memory (T2+CD27+IgM−), and IgM memory (T2+CD27+IgM+), we tested the effect of B cell-derived supernatants from sorted B-cell subpopulations on the proliferation of anti-CD3/28 stimulated T cells. The data reveal that the cytokines preserved in the B-cell supernatants are not sufficient to promote the suppression of T-cell proliferation (Fig. 5B and C). Since the suppressive effect is also observed in B-cell cocultures with purified T-cell subsets, this implies that the regulatory effect probably requires physical B cell–T cell interaction. At this stage, we can only hypothesize that surface molecules mediating this interaction could be required for the suppressive function. Future experiments will be needed to elucidate the cellular requirements as well as role of TNFR2 signaling in this process.

Notably, in the purified B-cell cultures the levels of IL-10 detected in the supernatants were generally low (Fig. 5C) and...
Figure 6. Selection and formation of Breg in the context of a memory response. (A) Terminal B-cell differentiation. I. Activation with TLR9 ligand (TLR9L) CpG DNA selects memory B cells (Bm) for terminal differentiation. II. Formation of Bregs from Bm in the transition process to antibody-secreting cells. CD27-(naïve) B cells (Bn) do not differentiate to Bregs in this experimental setting. Bregs secrete IL-10 and other suppressive cytokines and express TNFR2 along with costimulatory molecules. III. Loss of TNFR2 accompanies the differentiation into antibody-secreting cells (ASC). (B) Bregs guide T-cell differentiation. Bregs exert their effects on T cells via secretion of IL-10 and other soluble mediators and via cell-cell contact mediated by costimulatory receptors (Co-R) expressed on T and B cells and possibly by interaction of membrane TNF on T cells with TNFR2 on B cells. I. Breg arising from memory B cells (Bm) dampen antigen presentation and block naïve T-cell priming, II. block T-cell proliferation and, subsequently, III. influence Th polarization.

differences among the individual B-cell subpopulations were not statistically significant in this setup. However, this could be due to the time point of supernatant collection and the low density of B cells per well. We can further speculate that this effect could also indicate that in the cocultures with T cells and PBMC, B cell-derived IL-10 is either more stable or prestimulated IL-10 producing B cells augment IL-10 production by T cells and other leukocytes present in the cocultures although IL-10 levels remained low in the absence of supplemented B cells (Fig. 3C and 4B).

Taken together, this study demonstrates that expression of TNFR2 on human peripheral blood B cells coincides with IL-10 production and regulatory function. More detailed analyses revealed that TNFR2 expression is a characteristic of memory B-cell subsets that share the capacity to interfere with T-cell expansion. Most notably, the latter effect was not reproducible with soluble mediators derived from TLR9-stimulated B-cell subpopulations (supernatants), highlighting a role of cell–cell contact in TNFR2+ B cell-mediated T-cell suppression. Follow-up studies still need to explore the signaling pathways underlying the distinct functional properties of the two TNFR2+ memory B-cells subsets. Nevertheless, our data suggest that the frequency and distribution of TNFR2+ B cells in patients could represent a promising new parameter for immunomonitoring of resolution of infection or activity of autoimmune disease.

Materials and methods

Cell isolation and stimulation

PBMC were isolated from buffy coats from healthy donors obtained from German Red Cross South transfusion center (Frankfurt am Main, Germany). The use was approved by the local ethics committee (Approval #154/15). PBMC were isolated by Pansoll gradient centrifugation (PAN-Biotech, Aidenbach, Germany) followed by B-cell positive selection with anti-CD19 microbeads (MiltenyiBiotec, Germany). Purity was controlled by flow cytometry and was ≥97%. Isolated cells were cultivated in RPMI 1640 (Gibco, Life science, Darmstadt, Germany) supplemented with 10% FCS (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1% penicillin/streptomycin, 1% L-Glutamine, and 1%
HEPES buffer (all from Biochrom, Berlin, Germany). Cells were seeded at a concentration 10^6 cells/mL (if not stated differently) and cultivated in 96-well plates (Greiner CELLSTAR® round bottom; Greiner Bio-One, Kremsmünster, Austria). All cells were cultivated in a 5% CO2 incubator at 37°C. For stimulation, 1 μM full-length PTO-modified CpG 2006 (5′-tcgtcgttttgtcgttttgtcgtt-3′) (Eurofins MWG Biotech, Germany) and/or UV-irradiated BHK-CD40L (CD40L) and BHK-pTCF (pTCF) (1:10) were applied.

Cell sorting

B cells were stimulated as indicated for 2 days. Supernatant was removed, cells were washed, check for viability using trypan blue (Applichem Panreac, Darmstadt, Germany), and counted (TC20 Automated Cell Counter; Bio-Rad). Staining of human B-cell subsets before sort was performed with the following antibodies: anti-CD19-PE-Cy7 (Beckman Coulter), anti-CD27-BV421 (BD Biosciences, Heidelberg, Germany), anti-IgM-BV605 (BioLegend), anti-TNFR2 (CD120b)-APC (R&D Systems, Inc.), and murine IgG2A-APC (R&D Systems, Inc.) as isotype control. Cells were incubated in the dark for 30 min at 4°C in PBS with 0.5% FCS. B-cell subpopulations were sorted on a FACSARia™ Fusion (BD Biosciences) using the version 8.0.1. of the BD FACS Diva software. Purity of sorted subpopulations was confirmed by remeasuring of samples. Sorted cells were washed, counted, and checked for viability using trypan blue.

Proliferation assay

Autologous PBMC without B cells (CD19-; PBMCΔB) were kept in an incubator in presence of 12 ng/mL IL-2 (Miltenyi Biotec, Germany) for 2 days. For experiments with CD4 cells only — the cells were obtained by positive selection from PBMC with anti-CD4 microbeads (Miltenyi Biotec) on day 2. Proliferation of T cells was assessed using CFSE staining. PBMC were washed and stained in 1 μM solution of CFSE (eBioscience) for 10 min at room temperature, staining was stopped with addition of FCS and cells were washed three times with cold RPMI containing 10% FCS in a precooled centrifuge to remove unbound CFSE. Stained cells were seeded (2.5 × 10^5/mL), stimulated with CD3/28 beads (T-cell Activation/Expansion Kit; Miltenyi Biotec) in ratio 1:2 (bead to cells) and cocultured with B cells (5 × 10^5/mL) in ratio 1:2 (T cell:B cell). IL-10 (Miltenyi Biotec) was used in concentration (10 ng/mL).

Quantification of CFSE dilution by flow cytometry was measured on day 4. T cells were identified with anti-CD4 PerCP (BD Biosciences) and anti-CD8 (BD Biosciences) antibodies. Samples were acquired using a FACS LSRII SORP (BD Biosciences) and cytometry data (LMD files) were analyzed with Kaluza software (Beckman Coulter). The aqua fluorescent reactive dye (LIVE/DEAD Fixable dead Cell stain Kit, Invitrogen) was used for definition of live and dead cells in all experiments. Doublet exclusion was applied for all measurements. The flow cytometry gating strategy for evaluation of proliferation of CD4- and CD8- T cells is shown in Supporting information Fig. S1.

Supernatant from B cells used for coculture experiments was prepared as a mixture 1:1:1:1 from supernatants of B-cells subpopulations of four independent donors. These supernatants were collected from B-cells subpopulations cultivated for 4 days at 100,000 cells/well. A total of 100 μL of supernatant mixture was added to the stimulated PBMC.

CVID patients and healthy donors

PBMC from 22 CVID patients and 11 healthy donors were studied for TNFR2 expression on peripheral B cells after 3 day stimulation with 1 μM CpG 2006 ODN (BIOMOL). Cell isolation, cryopreservation and cultivation conditions were done according to protocol published in [36]. The use of patient cells in this study was approved by the Ethics committee of St Anne's University Hospital in Brno (No. 11G /2009). Blood samples were collected after informed consent was signed by the participants.

ELISA

Supernatants were collected from cells on day 4 of coculture and IL-10 was quantified by ELISA (Human IL-10 ELISA Set, BD Bioscience).

Statistical analysis

Statistical analysis of results was carried out using GraphPad Prism 7.01 (GraphPad Software Inc.). Data were analyzed using repeated measures one-way ANOVA with Geisser-Greenhouse correction and Tukey’s correction for multiple comparisons tests, two-tailed Wilcoxon matched-pairs signed rank test, or Friedman test. For multiple comparisons all-against-all conditions containing B-cell fractions were included. Statistically significant p-values are listed in the graphs or symbols representing p values are used as follows: *p < 0.05, **p < 0.01, ns, nonsignificant.

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