Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells

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Running title
GRANZYME B⁺ REGULATORY B CELLS INFILTRATE TUMORS

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Abstract

The pathogenic impact of tumor-infiltrating B cells is unresolved at present, however, some studies suggest that they may have immune regulatory potential. Here we report that the microenvironment of various solid tumors includes B cells that express granzyme B (GrB, GZMB), where these B cells can be found adjacent to IL-21-secreting T regulatory cells (Tregs) that contribute to immune tolerance of tumor antigens. Because Tregs and plasmacytoid dendritic cells are known to modulate T effector cells by a GrB-dependent mechanism, we hypothesized that a similar process may operate to modulate B regulatory cells (Bregs). IL-21 induced outgrowth of B cells expressing high levels of GrB, which thereby limited T cell proliferation by a GrB-dependent degradation of the T cell receptor ζ-chain. Mechanistic investigations into how IL-21 activated GrB expression in B cells to confer Breg function revealed a CD19⁺CD38⁺CD1d⁺IgM⁺CD147⁺ expression signature, along with expression of additional key regulatory molecules including IL-10, CD25 and IDO. Notably, induction of GrB by IL-21 integrated signals mediated by surface IgM (BCR) and Toll-like receptors (TLR), each of which were enhanced with expression of the B cell marker CD5. Our findings show for the first time that IL-21 induces GrB⁺ human B regulatory cells. They also establish the existence of human B cells with a regulatory phenotype in solid tumor infiltrates, where they may contribute to the suppression of anti-tumor immune responses. Together, these findings may stimulate novel diagnostic and cell therapeutic approaches to better manage human cancer as well as autoimmune and graft-versus-host pathologies.
Introduction

It is widely recognized that certain B cell subpopulations exhibit potent regulatory properties and are involved in immune pathologies including autoimmune and malignant diseases(1, 2). In murine autoimmune models, adoptive B cell transfer can ameliorate chronic inflammatory responses even when the disease is already established(1, 3). Similarly, in human patients with rheumatoid arthritis, disease activity is lower in patients with high peripheral B cell counts(4). One of the factors produced by regulatory B cells (B_{reg}) is the immunosuppressive cytokine IL-10, which has recently been identified in mouse models(1, 5-7) and humans(1, 2, 8, 9). Nevertheless, although some clinical observations clearly support the concept of human B cells contributing to the limitation of autoimmune diseases, in other cases they are known to have rather aggravating potential(10). To better understand the development of regulatory versus pathogenic B cells, a more comprehensive knowledge of factors inducing and mediating regulatory B cell functions is necessary.

A previously discovered cytokine with pleiotropic effects on a variety of immune cells is interleukin 21 (IL-21)(11). The effects of IL-21 on B cells strongly depend on additional signals including TLR agonists, B cell receptor (BCR) stimulation and CD40 ligation(12). Recently, CD40 ligand (CD40L) was shown to determine whether IL-21 induces differentiation of B cells into plasma cells(13, 14), or, in its absence, into B cells secreting the serine protease granzyme B (GrB)(15, 16). Importantly, GrB is characterized not only by its classical function as cytotoxic protease, but also exhibits immunosuppressive properties such as in regulatory T cells (T_{reg})(17-19) and dendritic cells(20). In line with these findings, IL-21 has been described to mediate expression of IL-10 by murine cytotoxic cells and B cells (21, 22), supporting its involvement in immune regulation.

Several inflammatory conditions including systemic lupus erythematosus (SLE) and acute viral infections are characterized by both elevated GrB(23, 24) and IL-21 serum levels(15, 25, 26). Moreover, we recently demonstrated that GrB^{+} B cells can be found in SLE patients(15) and in subjects vaccinated against viral infections(16). An association of GrB^{+} B cells with solid tumors has not been reported so far. However, while infiltration of certain tumors with B cells can be associated with a good prognosis(27-30), in others the presence of B cells support a tumor-protective environment(31-33). We therefore hypothesized that GrB-expressing B
cells may phenotypically represent B<sub>reg</sub> and may be present in the microenvironment of human tumors.

In the current work we demonstrate that IL-21 induces a regulatory phenotype in human B cells with expression of immunoregulatory molecules including GrB, IL-10, indoleamine-2,3-dioxygenase (IDO) and CD25. Moreover, we show that GrB<sup>+</sup> B cells suppress T cell proliferation by GrB-dependent degradation of the T cell receptor ζ-chain, a known GrB substrate(34). Importantly, we demonstrate that GrB<sup>+</sup> B cells as well as IL-21-providing T cells are present in the tumor microenvironment of certain solid tumors. Of note, CD5<sup>+</sup> B cells from human cord blood possess an enhanced capacity to express GrB, supporting the concept that CD5<sup>+</sup> B cells may play a particular role in immune regulation, as suggested by various mouse models(5-7).

In summary, our findings suggest IL-21 is a key cytokine for the generation of human regulatory B cells under certain environmental contexts, and GrB represents a central immunomodulatory molecule expressed by such B<sub>reg</sub>. GrB<sup>+</sup> B<sub>reg</sub> may play a so far unappreciated role in human pathologies by infiltration of tumors or by modulation of inflammatory processes. Our study also suggests IL-21-induced B<sub>reg</sub> may prove useful as innovative cell-therapeutic tool for the management of undesired immune activation in autoimmune and graft-versus host diseases.
Materials and Methods

Human subjects and cell culture
The use of blood from human subjects was approved by the Ethics Committee at Ulm University. Blood samples were collected after informed consent had been given. Peripheral blood mononuclear cells (PBMC) or mononuclear cells from umbilical cord blood (CBMC) were isolated by Ficoll density gradient centrifugation. CD19+ B cells (>99% purity) and CD4+ T cells, (>95% purity) were magnetically purified using appropriate negative selection kits according to the manufacturer’s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were suspended in AIM-V medium (Gibco BRL, Grand Island, NY, USA) and incubated on U-bottom 96-well plates at 1x10^6 cells/µl and 200µl/well, if not stated otherwise. For B-cell/T-cell co-culture experiments, CD4+ T cells were added to CD19+ B cells at a 1:1-ratio and incubated for the time indicated. Reagents used for functional assays are outlined in the manuscript supplement.

Reagents for functional assays
For BCR stimulation, affinity purified rabbit F (ab')2 against human IgA+IgG+IgM (H+L) was used at 6.5µg/ml (anti-BCR, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Human recombinant IL-21 (50ng/ml) was purchased from BioSource (Camarillo, CA, USA). Human recombinant IL-2 (100IU/ml) was obtained from PeproTech GmbH (Hamburg, Germany). CpG ODN 2243 was obtained from Coley Pharmaceutical Group (Wellesley, USA). IRS 661 and IRS 869 were purchased from Biomers (Ulm, Germany). Cycloheximide and Brefeldin A (both at 1 µg/ml) were from Sigma-Aldrich (Schnelldorf, Germany). TBEV antigens were used as inactivated TBEV vaccine (standard concentration 100ng/ml, strain Neudörfl) adsorbed to 0.35mg Al (OH)3 (FSME-IMMUN Erwachsene, Baxter, Heidelberg, Germany). For TCR stimulation, anti-CD3/CD28 antibody-coated beads (0.02µl/200µl-well) from Dynal (Invitrogen, Carlsbad, CA, USA) were used. For GrB inhibition, GrB inhibitor IV (Ac-IEPD-CHO) at 5µM (American Peptide Company, Sunnyvale, CA, USA) or a carrier- and preservative-free goat anti-human GrB polyclonal antibody (IgG) at 10µg/ml (R&D Systems, Minneapolis, MN, USA) were used.

Flow cytometry
FITC-, PE-, PE-Cy5-, PE-Cy7- or APC-labeled antibodies (ab) to CD1d, CD3, CD4, CD5, CD19, CD20, CD25, CD27, CD38, CD58, CD69, CD70, CD86, CD107a, IgD and IgM were
purchased from Becton Dickinson (BD) Biosciences (Heidelberg, Germany). FITC-labeled ab to CD10 and PE-labeled abs to CD24 were purchased from EXBIO (Praha, Czech Republic), FITC-labeled abs to CD58, CD147, Annexin V and PE-labeled abs to IL-10 from Immunotools (Friesoythe, Germany), FITC-labeled abs to CD154 from BioLegend (San Diego, CA, USA), Alexa Fluor 488-labeled abs to human IDO from R&D Systems (Minneapolis, MN, USA), carboxyfluorescein succinimidyl ester (CFSE) from Sigma-Aldrich (Schnelldorf, Germany) and PE- and APC-labeled abs to human GrB (clone GB12) from Invitrogen (Carlsbad, CA, USA). Appropriate isotype controls were used. For intracellular GrB, IL-10 and IDO staining, cells were incubated with brefeldin A (1 µg/ml) during the last 4h of incubation and analyzed as recently described(16). For apoptosis detection, cells were stained with Annexin V for 15min at room temperature (RT). Then, PI (1mg/ml) was added and samples immediately analyzed by FACS. Flow cytometric analyses were performed on a FACScan or a FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA). Data were analyzed using FlowJo (version 9.3.1; Tree Star, Stanford, CA, USA).

**CFSE staining and proliferation assay**

For proliferation experiments, 1x10^7 cells purified CD4^{+} T cells were resuspended in 10 ml PBS containing 0.1% BSA (PBS/BSA). CFSE was added to a final concentration of 1µM and cells were incubated at 37°C for 10min. Incubation was stopped by adding 40ml ice cold culture medium and suspension was incubated for 5min on ice before 3 additional washing steps with PBS/BSA. Then, CD4^{+} T cells were co-cultured for 6 days with autologous or allogenic purified B cells in the presence of anti-CD3/28 beads, IL-2, IL-21, anti-BCR, GrB inhibitors or combinations of these as indicated. After 3 days of culture, 100µl medium was exchanged.

**GrB ELISpot**

Human GrB ELISpot kits were purchased from Gene-Probe Diaclone SAS (Besançon Cedex, France) and PVDF-bottomed 96-well plates from Millipore (Bedford, MA, USA). ELISpot assays were performed as recently described(15). Briefly, cells were plated in AIM-V medium at 1x10^5 per 100µl per well for 16h. Then, plates were developed, read on an Immunospot Series 1 Analyzer and spots counted using Immunospot 3 software (CTL Cellular Technology Ltd., Cleveland, OH, USA).

**ELISA for enzymatically active GrB**
For the detection of enzymatically active, secreted GrB in the supernatants of stimulated B cells we used a highly specific GrB activity assay according to the manufacturer’s protocol (SensiZyme, Sigma-Aldrich, Munich, Germany).

**Spinning disk confocal microscopy**

Purified CD19⁺ B cells were cultured for 16h in the presence of IL-21 and anti-BCR. Purified CD4⁺ T cells at 1x10⁶ cells/ml were cultured separately in the presence of anti-CD3/28 beads. Then, 1x10⁵ T cells were harvested, put on ibiTreat chamber slides (ibidi GmbH, Martinsried, Germany) for 30min for immobilization, stained with Cell Mask deep red membrane dye (Invitrogen, Carlsbad, CA, USA) at 5µg/ml for 15min at 37°C and washed three times with PBS. 1x10⁵ B cells in 50µl of PBS and 25µl of GranToxiLux fluorogenic GrB substrate (OncoImmunin, Gaithersburg, MD, USA) were added. Fluorescence images were acquired using a spinning disk confocal microscope and the acquisition software Andor iQ 1.6 as described previously(20).

**Western immunoblotting**

Purified B cells (>99%) from healthy individuals were stimulated with anti-BCR ± IL-21 overnight. Autologous purified CD4⁺ T cells (>98%) were simultaneously stimulated with anti-CD3/28 beads in a separate culture. After removal of beads and treatment with 1µg/ml cycloheximide, CD4⁺ T cells were co-cultured at a 1:1-ratio with prestimulated B cells for 24h in the presence or absence of anti-GrB antibodies. Then, cells were resuspended in lysis buffer (10mM HEPES, 10mM NaCl, 1mM KH₂PO₄, 5mM NaHCO₃, 1mM CaCl₂, 0.5mM MgCl₂, 5mM EDTA in A. dest.) containing a protease inhibitor (protease inhibitor cocktail set III, Calbiochem, Darmstadt, Germany) for 15min on ice. Protein content was measured using Pierce BCA colorimetric protein assay kit (Thermo Scientific, Rockford, IL, USA). Then, proteins were separated on 18% SDS-polyacrylamide gels and transferred onto 0.45µm PVDF membranes. After washing and blocking, membranes were incubated with primary monoclonal abs against human TCR-ζ (16h, 4°C; Biolegend, San Diego, Ca, USA) or β-actin (30 min, RT; Thermo Scientific, Rockford, IL, USA). Then, membranes were washed 3 times and incubated with an HRP-conjugated secondary ab (goat anti-mouse IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1h at RT. Finally, membranes were developed and band intensities quantified using Image J.

**Cytometric bead array (CBA)**
For quantification of IL-2, supernatants from B cells and T cells co-cultured at a 1:1-ratio for 3 days were analyzed using a BioPlex-9 Plex Cytokine Assay Kit (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. All incubations were performed at RT. Briefly, the anti-cytokine bead solution was diluted and 50µl were placed on 96-well Durapore membrane plates (Millipore, Billerica, MA). After washing by vacuum filtration, 50µl of undiluted supernatants and standards were placed into wells and incubated for 30min on a shaker. After washing, 25µl detection ab was added and incubated for another 30min while shaking. After washing, streptavidin–PE was added for 10min. Finally, plates were washed, resuspended in assay buffer and read on a flow cytometer (Luminex, Riverside, CA). Data were analyzed using Bio-Plex Manager software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence microscopy
Formalin-fixed, paraffin-embedded cancer tissue sections (1µm) from various solid epithelial cancers including breast, cervical, ovarian, colorectal and prostate carcinomas were obtained from the local department of Pathology (Table 1). In compliance with the German law for correct usage of archival tissue for clinical research the blocks were anonymized. Double immunofluorescent staining of formalin-fixed, paraffin-embedded cancer tissue sections against GrB, IL-21 or IL-10 as first antigens and against CD19 or CD3 as second antigens was performed as follows: Tissue sections were deparaffinized in xylol and alcohol and antigens retrieved by 20min incubation in 10mM citric acid (pH 6.0) in a pressure cooker. Then, sections were incubated with primary antibodies (abs) against GrB (mouse anti-human GrB, Dako Cytomation, Glostrup, Denmark), IL-21 (rabbit anti-human IL-21, Acris Antibodies, San Diego, CA, USA) or IL-10 (rabbit anti-human IL-10, Acris Antibodies, San Diego, CA, USA) for 30min in a moist chamber and rinsed with PBS. Subsequently, sections were incubated with secondary biotinylated abs (anti-mouse or anti-rabbit, Dako Cytomation, Glostrup, Denmark) for 30min. For GrB and IL-21 staining, this was followed by a 30min incubation with a streptavidin-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR, USA), for IL-10 staining with a DyLight 488-conjugated anti-rabbit secondary antibody (Dianova, Hamburg, Germany). To enable double immunohistochemistry with abs from the same host as used for the first antigen, sections were placed on a heating plate for 4min at 90°C in H2O to denature open binding sites on the primary abs. Then, slides were incubated with primary mouse anti-human CD19 or CD3 abs (Dako Cytomation, Glostrup, Denmark) for 30min, followed by incubation with a Cy3-conjugated anti-mouse ab (Dianova, Hamburg, Germany) for 30min. Internal controls included exclusion of nonspecific binding of
Cy3-conjugated secondary abs as well as premature denaturation of ab binding sites by placing the heating step directly after binding of the primary anti-GrB and anti-IL-21 abs respectively. In this case, no binding of secondary biotinylated abs could be observed. For analysis, nuclei were stained with DAPI, sections were mounted in Vectashield mounting medium (Vector Laboratories Inc, Burlingame, CA, USA) and visualized using an Axioscope 2 fluorescence microscope (Zeiss, Göttingen, Germany).

Statistics
Data are expressed as mean values ± SEM unless stated otherwise. Statistical differences between the means of two data columns were assessed using the unpaired and paired Student’s t-test as appropriate. Results with p-values <0.05 were considered statistically significant. p-values were corrected using the Bonferroni method where applicable.
Results

Interleukin 21-activated B cells produce granzyme B and suppress CD4⁺ T cell proliferation.

Recently, we found that the IL-2 family cytokine IL-21 can trigger B cells to secrete the serine protease GrB (15, 16). Since expression of GrB by B cells is not accompanied by perforin expression, they may exhibit perforin-independent functions (23), such as the immunosuppressive capacity of Treg (17-19). To test the hypothesis that GrB⁺ B cells may exhibit similar functions, we isolated B cells and pre-activated them with IL-21 and BCR stimulation. IL-21 rapidly induced expression and secretion of enzymatically active GrB in a number of B cells, an effect strongly enhanced by simultaneous BCR stimulation (Fig. 1A, B & Suppl. Fig. 1). We then isolated CD4⁺ T cells and induced proliferation using by activating CD3 and CD28 (CD3/CD28). Subsequently, we incubated CD3/CD28-stimulated T cells for 6 days in the presence or absence of differentially activated B cells. T cell proliferation was strongly suppressed in the presence of IL-21/anti-BCR-activated B cells, but not in the absence of B cells (Suppl. Fig. 2) or in the presence of unstimulated or IL-2-stimulated B cells (Fig. 1C, D). B cells stimulated with IL-21 alone had a mild suppressive effect only, corresponding with a lower expression of GrB (Fig. 1C, D). Both a GrB-neutralizing antibody and a specific GrB substrate inhibitor were able to rescue T cell proliferation (Fig. 1E, F). The lower proliferation of T cells in the presence of BCR-stimulated B cells is due to enhanced consumption of media with anti-BCR-induced B cell proliferation.

Inhibition of T cell proliferation by B cells involves transfer of active GrB to T cells and degradation of the T cell receptor ζ-chain, but not induction of T cell apoptosis.

Next, we characterized the underlying interactions between GrB⁺ B cells and T cells. First, we tested whether B cell-derived GrB is delivered to T cells in its active form. To this end, we pre-incubated B cells with or without IL-21 and anti-BCR and then started co-incubating them with CD4⁺ T cells in the presence of a GrB-specific fluorogenic substrate on microscopy dishes. Spinning disk confocal microscopy demonstrated that after 2-3h T cells began to internalize B cell-derived GrB into the cytoplasm (Fig. 2A & Suppl. Video 1). No active GrB was observed in T cells in the presence of unstimulated, GrB⁻ B cells (data not shown).

A potential impact of GrB on T cells may be induction of apoptosis by caspase cleavage. Nevertheless, using FACS analysis we excluded that B cells stimulated in the presence of IL-
21 ± anti-BCR were able to induce significant T cell apoptosis (Suppl. Fig. 3A, B). A more recently identified GrB substrate is the ζ-chain of the T cell receptor (TCR-ζ), which plays a role in delivering activation and growth signals to T cells(34). To test the impact of B cell-derived GrB on TCR-ζ we co-incubated CD3/CD28-activated CD4+ T cells in the presence or absence of B cells activated with IL-21 ± anti-BCR. B cells stimulated with IL-21 and anti-BCR, but not B cells stimulated with anti-BCR only, were able to strongly suppress TCR-ζ expression in co-incubated CD4+ T cells (Fig. 2B, C). This effect was not seen in the absence of B cells (data not shown). More importantly, TCR-ζ expression in T cells was partly rescued by a GrB-neutralizing antibody (Fig. 2B, C).

**GrB expression by IL-21-stimulated B cells is part of a regulatory B cell phenotype.**

As outlined above, we primarily described IL-21-induced B_{reg} as cells able to express and secrete GrB (Fig. 1), a capacity they appear to share with regulatory T cells(17-19) and regulatory dendritic cells(20). To define in more detail the phenotype of IL-21-induced GrB⁺ B cells, we tested a variety of surface antigens described in the past to characterize B_{reg}(5, 6, 8, 9, 35). These antigens include activation markers, molecules of the immunoglobulin superfamily, costimulatory molecules, enzymes and adhesion molecules. We found significant upregulation of several of these molecules in IL-21-stimulated GrB⁺ B cells including CD38, CD1d, IgM, CD86, CD154, CD10 and CD20 (Fig. 3). CD70 showed a higher expression in GrB⁺ B cells as compared to unstimulated B cells, while CD24, CD27 and IgD exhibited no increase or even a decrease in GrB⁺ B cells (Fig. 3).

A frequently reported characteristic of B_{reg} is the expression of IL-10, with such B cells often referred to as B10 cells(2, 5, 6). Here, we demonstrate that healthy peripheral B cells stimulated with IL-21/anti-BCR also responded with the development of a small but significant population of B cells expressing IL-10 (Fig. 4A, B). This population did not occur in the presence of IL-2 nor in the absence of cytokines. Moreover, co-stimulation of B cells with IL-21/anti-BCR was able to induce IDO, another regulatory molecule previously described in dendritic cells(36). Again, this effect was small but significant, and most pronounced in the presence of IL-21, but not with IL-2 or without cytokines (Fig. 4C, D). Importantly, after stimulation of B cells with IL-21 and anti-BCR, GrB not only was the first regulatory molecule expressed (Fig. 1), but all additional regulatory molecules tested were primarily expressed on GrB⁺, but not on GrB⁻ B cells (Suppl. Fig. 4).
Since IL-21-activated GrB+ B cells appear to express characteristic B<sub>reg</sub> markers, we also tested T<sub>reg</sub>-specific antigens. Regulatory T cells typically express high levels of CD25 and it was hypothesized that part of their inhibitory effect is effector T cell deprivation of IL-2 (37, 38). IL-21-stimulated B cells indeed significantly upregulated surface CD25 (Fig. 4E, F), and were able to capture free IL-2 from T cell/B cell co-cultures (Fig. 4G). Nevertheless, we were not able to rescue the proliferative response of co-cultured T cells by retitrating recombinant IL-2 (Suppl. Fig. 5), indicating that IL-2 deprivation may not be the primary mechanism exhibited by human B<sub>reg</sub>. Finally, IL-21-activated B cells expressed increased levels of the T<sub>reg</sub>-specific Ig family member CD147 (39) (Fig. 3), whereas neither Foxp3 nor CTLA-4 or CD28 were expressed by IL-21-stimulated B cells (data not shown).

**CD5+ B cells exhibit an enhanced potential to express GrB as compared to CD5− B cells.**

CD5 is another marker described on murine B<sub>reg</sub> (5, 6), although it is not expressed on IL-21-induced GrB+ peripheral B cells (data not shown). Recently however, we found that CD5+ B cells from patients with SLE constitutively express GrB, in contrast to CD5− B cells from normal healthy donors (15). To directly compare the GrB potential of CD5+ versus CD5− B cells, we isolated B cells from cord blood samples and from adult healthy donors. We found that the GrB response of CD5+ B cells to IL-21 was more than 2-fold stronger than the response of CD5− B cells from healthy adult donors (Fig. 5A, B, C). Moreover, we previously showed that B cells from healthy volunteers vaccinated against viral infections such as tick-borne encephalitis virus (TBEV) respond with stronger GrB expression to IL-21 stimulation than B cells from unvaccinated donors (16). Therefore, we compared the GrB response of CD5+ and CD5− B cells from normal donors to IL-21 in the presence of TBEV vaccine instead of anti-BCR. Here, the GrB response of CD5+ B cells was up to 10-fold stronger (Fig. 5D, E), suggesting an innate potential of CD5+ B cells to express GrB in the presence of danger signals like viral stimuli.

**IL-21-induced GrB expression by B cells depends on both BCR and TLR signaling pathways**

IL-21-induced GrB expression by peripheral B cells is strongly enhanced in the presence of BCR stimulation, an effect suppressed by SYK inhibition (16). However, the induction of GrB in antigen-unexperienced CD5+ B cells by viral antigens (Fig. 5D, E) suggests the additional involvement of toll-like receptors (TLR) like TLR7 or TLR9. Indeed, we found that inhibition of both TLR7 and TLR9 using specific inhibitory ODN(40) suppressed IL-21-
induced GrB expression in B cells in a dose-dependent manner. This effect was found in both CD5− and CD5+ B cells (Suppl. Fig. 6).

**GrB-expressing B cells and IL-21-expressing T cells reside in the microenvironment of various solid tumors**

Although the regulation of immunity is important, its suppression may be undesired when the establishment of a robust immune response, for example against a tumor, is required. To evaluate whether B cells with a regulatory phenotype could play a role in the modulation of anti-tumor immune responses, we screened a variety of tumor tissue sections for GrB+ B cells. We found that GrB-expressing B cells reside within the microenvironment of different tumor types including breast, ovarian, cervical, colorectal and prostate carcinomas (Fig. 6A-F & Table 1). Since we identified IL-21 as key cytokine for differentiation of B cells into GrB+ B cells, we tested whether IL-21-expressing cells are also present in the tumor tissue. As expected, IL-21-expressing CD3+ T cells could be identified in the vicinity of B cells in these tissue sections (Fig. 6G, H). Importantly, we also detected IL-10-expressing CD19+ B cells in the same tumor tissues that contained GrB+ B cells, suggesting that B cells with a regulatory phenotype in this tumor microenvironment may express both IL-10 and GrB (Suppl. Fig. 7). Nonetheless, the frequency of IL-10+ B cells in tumor tissue was significantly lower than the frequency of GrB+ B cells, which is in line with our in-vitro data showing that the predominant regulatory molecule expressed by IL-21-induced human Breg is GrB (Suppl. Fig. 4).
Discussion

B cell-mediated immune regulation appears to be a fundamental property of the immune system\(1, 2\). In the present study we identified IL-21 as key cytokine for the induction of human regulatory B cells. Their phenotype includes surface markers like CD1d, CD38, IgM, CD10, CD86 and CD154\(5, 6, 8, 9, 35\), but also molecules with actual regulatory functions including GrB\(17-20\), IL-10\(2, 5, 6\), IDO\(36\) and CD25\(37, 38\). IL-21 is known to have a variety of effects on B cells, depending on their maturation stage and the presence of further co-stimulatory signals. In certain cases, IL-21 can induce B cell proliferation, survival, differentiation into plasma cells, or isotype switching\(13, 14\). In other situations, B cells rather undergo apoptosis and cell cycle arrest after IL-21 stimulation\(41\). Recently, CD154 (CD40L) was identified as important determinant for IL-21-induced differentiation of human B cells into either plasma cells\(13, 14\) or into GrB-secreting B lymphocytes\(15, 16\). Here, we show that human B cells gain regulatory potential in response to IL-21, provided additional triggering of the BCR and TLRs is present. Although their involvement in the development of human B\(_{\text{reg}}\) has been proposed before\(1, 42\), a specific role of IL-21 in this regard has not been considered so far.

The exact phenotype of B\(_{\text{reg}}\) is not clearly defined and multiple discrepancies exist between different studies\(1, 2\). A common finding in mice appears to be IL-10 secretion and expression of CD1d and CD5\(5-7\). These data were only partly confirmed in humans\(8, 9\). A recent study described a human CD19\(^+\)CD24\(^{hi}\)CD38\(^{hi}\) B cell population with regulatory potential, which is functionally impaired in SLE patients\(8\). This B cell population secreted IL-10, but did not express CD5. Of note, its regulatory capacity was dependent on CD86 expression, but only partially on IL-10 secretion, so that the authors postulated the existence of further soluble factors with regulatory potential. These results are complementary to our data showing that CD86 is strongly upregulated in IL-21-activated B\(_{\text{reg}}\) and that the B\(_{\text{reg}}\) identified in our study secrete several additional regulatory molecules. One of these molecules is CD25, which is also expressed on T\(_{\text{reg}}\)\(37, 38\). A recent study demonstrated the development of large, CD25\(^+\) B cells with regulatory potential after polyclonal activation with different TLR-agonistic stimuli\(43\). Part of the regulatory effect identified in CD25-expressing B cells may therefore be due to IL-2 deprivation\(37, 38\). We further identified CD147 to be upregulated by IL-21-induced B\(_{\text{reg}}\), an immunoglobulin family member that was recently postulated as novel marker for highly active CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T\(_{\text{reg}}\)\(39\). Although
further molecules typically associated with T_{reg}, including CTLA-4 and Foxp3, were not expressed by IL-21-induced B_{reg}, the panel of regulatory molecules compiled in this study including GrB, IDO, IL-10 and CD25 suggests mechanistic similarities between GrB-expressing B_{reg} and T_{reg}. Given previous findings in T_{reg} and in pDC(17-20), we hypothesize that particularly GrB may be a common effector molecule of human regulatory cells in general. Apart from the GrB effect identified in our study, namely TCR-ζ degradation, GrB may exhibit further regulatory functions such as TGF-β release from soluble β-glycans(44) or induction of apoptosis in certain immune cells. In IL-21-induced human B_{reg} GrB not only is the first regulatory molecule expressed, but all other regulatory molecules tested are expressed primarily on GrB^{+}, but not on GrB^{-} B cells. This suggests that GrB represents a predominant regulatory molecule and an important novel marker of human B_{reg}.

Although the existence of tumor-infiltrating B cells has been previously described(27, 45), their significance for solid tumors remains unclear. For example, in ovarian cancers some publications correlate the presence of B cells in the tumor microenvironment with a better prognosis(28), whereas others state that their presence is associated with a worse outcome(33). In order to detect the ,,smoking gun“ of B cells with a GrB^{+} regulatory phenotype directly in tumor tissue, we therefore started screening various human tumors for CD19^{+}GrB^{+} cells. This screening revealed that GrB-expressing B cells, IL-21-providing T cells, and some IL-10-expressing B cells indeed infiltrate solid tumor tissue. These findings, and recent data demonstrating down-regulation of the TCR-ζ chain in defined solid tumors(46), suggest IL-21-induced GrB^{+} B cells may indeed play an immunosuppressive role in certain tumors, possibly with similar impact on T cell responses as T_{reg}. Nevertheless, while the presence of IL-21^{+} cells provides a valid explanation for the induction of GrB^{+} B cells in tumor tissue, a variety of co-factors such as CD40L, TLR ligands and tumor-derived danger signals may influence the function of IL-21. Moreover, local secretion within a tumor may allow IL-21 to interact with different cell types than its systemic occurrence. It is therefore possible that IL-21 supports an immunosuppressive environment within a tumor, while its systemic application may allow activation of cytotoxic T cells and NK cells with anti-tumor activity at tumor-distant sites, eventually even resulting in the establishment of a robust anti-tumor immune response as recently described(47). Altogether, it likely depends on the activation status of infiltrating B cells, and the presence of additional co-factors whether an efficient anti-tumor immune response is suppressed or supported by infiltrating B cells.
In conclusion, we have shown for the first time that IL-21 is a key cytokine for the induction of human B_{reg} and have identified GrB as predominant immunomodulatory molecule and phenotypic marker of human B_{reg}. IL-21-mediated induction of GrB is significantly stronger in CD5^{+} as compared to CD5^{-} B cells, and integrates both BCR- and TLR-dependent signals. GrB plays a central role for the regulatory function of B_{reg}, since they can efficiently suppress T cell proliferation by GrB-dependent TCR-ζ degradation. GrB^{+} B cells exhibit a CD19^{-}CD38^{-}CD1d^{-}IgM^{-}CD147^{+} phenotype and express further regulatory molecules including IL-10, CD25 and IDO. Most importantly, GrB^{+} B cells can infiltrate the microenvironment of various solid tumors, where they are observed adjacent to IL-21-providing T cells. Our findings strongly suggest GrB^{+} B cells may contribute to the modulation of cellular adaptive immune responses by T_{reg}-like mechanisms, possibly allowing the escape of certain tumors from an efficient anti-tumor immune response. On the other hand, the induction and use of GrB^{+} B_{reg} may be evaluated as an innovative cell therapeutic approach to the management of immune pathologies including autoimmune and graft-versus-host diseases.
Acknowledgements

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Author contributions

References


Figure legends

Figure 1. Interleukin 21-activated B cells express granzyme B and suppress T cell proliferation.

(A-B) Healthy PBMC were cultured for 48h in the presence of indicated reagents. Cells were harvested, stained for CD19, fixed, permeabilized and stained for GrB. (A) Dot plots show GrB expression in CD19+ B cells from one representative experiment. Gates represent GrB+ B cells. (B) Bar graphs show mean percentages of GrB+ B cells from 5 individual experiments. (C-F) Healthy CD4+ T cells were stained with CFSE and co-cultured with B cells at a 1:1-ratio in the presence of IL-2, IL-21 anti-BCR, and GrB inhibitors as indicated. T cell proliferation was induced by anti-CD3/CD28 beads. After 6 days, cell cultures were harvested, stained for CD4 and analyzed by FACS. (C) Dot plots show CFSE-stained CD4+ T cells in the presence of allogeneic B cells prestimulated as indicated. Data are from one representative experiment out of 7 with similar results. Gated are proliferated T cells. (D) Bar graphs show mean percentages of proliferated T cells from 7 individual experiments. (E) Dot plots show CFSE-stained CD4+ T cells in the presence of autologous B cells prestimulated with anti-BCR ± IL-21 and GrB inhibitors as indicated. Proliferated T cells are gated. One representative experiment out of 3 with similar results is shown. (F) Bar graphs show mean percentages of proliferated T cells from 3 individual experiments.

Figure 2. B cells transfer active GrB to T cells and induce GrB-dependent degradation of the T cell receptor ζ-chain.

(A) Healthy B cells were isolated and activated using IL-21 and anti-BCR overnight. In parallel, CD4+ T cells were isolated and separately cultured with anti-CD3/CD28 beads. Then, T cells were stained with Cell Mask deep red membrane dye and co-cultured with B cells on a chamber slide. GrB-specific fluorogenic substrate was added for cellular detection of active GrB and live cell imaging performed for 6h. Shown are 9 video frames at different time points. Images are from one representative experiment out of 3 with similar results. (B) Purified B cells from healthy individuals were stimulated with anti-BCR ± IL-21 overnight. Autologous purified CD4+ T cells were simultaneously stimulated with anti-CD3/28 beads in a separate culture. After removal of beads and treatment with 1μg/ml cycloheximide, CD4+ T cells were co-cultured at a 1:1-ratio with prestimulated B cells for 24h in the presence or absence of anti-GrB antibodies. Then, cells were lysed, protein contents of samples equalized, and TCR-ζ protein determined by Western blot. β-actin was used as control. One
representative experiment out of 5 with similar results is shown. Control cultures with T cells and B cells cultured separately showed no change in TCR-ζ protein levels (data not shown). (C) Relative band intensities of TCR-ζ and β-actin were determined using Image J and ratios were calculated. Bar graphs show the ratios between TCR-ζ and β-actin band intensities.

**Figure 3. GrB⁺ B cells develop a regulatory B cell surface phenotype.**
Healthy PBMC were analyzed before or after culture with IL-21 and anti-BCR for 4 days. Cells were harvested, stained for CD19 and various surface markers, fixed, permeabilized, intracellularly stained for GrB and analyzed by FACS. Bar graphs show average MFIs from ≥3 individual experiments, dashed lines indicate average isotype controls.

**Figure 4. GrB induction in B cells by IL-21 is accompanied by upregulation of further immunoregulatory molecules including IL-10, IDO and CD25.**
(A-D) Healthy PBMC were cultured for 4 days with the reagents indicated. Then, cells were harvested, stained for CD19, fixed, permeabilized, intracellularly stained for IL-10 or IDO and analyzed by FACS. Isotype controls were negative in all experiments. (A) Zebar plots show IL-10 expression in CD19⁺ B cells from one representative experiment. Gated are IL-10⁺ B cells. (B) Bar graphs show mean percentages of IL-10⁺ B cells from 3 individual donors. (C) Zebar plots show IDO expression in CD19⁺ B cells from one representative experiment. Gated are IDO⁺ B cells. (D) Bar graphs show mean percentages of IDO⁺ B cells from 3 individual donors. (E-F) CD19⁺ B cells and CD4⁺ T cells from healthy individuals were isolated and co-cultured at a 1:1-ratio or cultured separately for 3 days in the presence of IL-21 and anti-BCR as indicated. Cells were harvested, stained for CD19, CD4 and CD25 and analyzed by FACS. (E) Line graphs show CD25 expression on B cells and T cells from one representative experiment. (F) Bar graphs show average CD25 expression on B cells after co-culture with CD4⁺ T cells from 3 individual experiments. (G) After a 3 day co-culture of B cells and CD4⁺ T cells as described above, supernatants were harvested and assayed for IL-2 by CBA. Bar graphs show average IL-2 concentrations in supernatants from 3 individual experiments.

**Figure 5. CD5⁺ B cells exhibit a higher potential to express GrB than CD5⁻ B cells.**
(A) Purified CD5⁺ cord blood B cells and peripheral CD5⁻ B cells from adult subjects were cultured on GrB-specific 96-well ELISpot plates at 10⁵ cells/well in the presence of IL-21 and anti-BCR as indicated. After 16h, plates were developed and dots counted. Each condition
was run in duplicates. Shown are representative ELISpot data from two individual experiments. (B-C) Purified CD5⁺ cord blood B cells and peripheral CD5⁻ B cells from adult subjects were cultured for 16h in the presence of IL-21 and anti-BCR as indicated, before being stained for intracellular GrB. (B) Dot plots show GrB expression in CD5⁺ and CD5⁻ B cells from two representative experiments. Gated are GrB⁺ B cells. (C) Bar graphs show mean percentages of CD5⁺ B cells from 3 cord blood donors and CD5⁻ B cells from 3 adult subjects. (D-E) Purified CD5⁺ cord blood B cells and peripheral CD5⁻ B cells from adult subjects were cultured for 16h in the presence of IL-21 and TBEV vaccine as indicated. Then, cells were intracellularly stained for GrB and analyzed by FACS. (D) Dot plots show GrB expression in CD5⁺ and CD5⁻ B cells from two representative experiments. Gated are GrB⁺ B cells. (E) Bar graphs show mean percentages of GrB⁺CD5⁻ B cells from 4 cord blood donors and GrB⁺CD5⁻ B cells from 4 adult subjects.

Figure 6. GrB⁺ B cells and IL-21-providing T cells infiltrate the microenvironment of solid tumors.

(A-F) Double immunohistochemistry (60-fold magnification) was performed on a series of formalin-fixed, paraffin-embedded cancer tissue sections from various tumors including breast (A-B), ovarian (C-D) and cervical (E-F) carcinomas. Tumor sections were co-stained with anti-CD19 ab (red), anti-GrB ab (green) and DAPI (blue, nuclear staining). Negative control stainings ruled out unspecific staining. (G) In alternatively stained tumor sections, IL-21-expressing cells (green) were detected adjacent to CD19⁺ B cells (red). (H) Finally, co-expression of IL-21 and CD3 was demonstrated by double immunohistochemistry of breast, cervical and ovarian carcinoma sections using anti-CD3 antibodies (red, surface staining) and anti-IL-21 antibodies (green).

Table 1. Characteristics of screened tumor sections.

Listed are clinical characteristics including tissue type, tumor characteristics, patient age, tumor stage and grade, as well as the frequency of CD19⁺ GrB⁻ cells and IL-21⁺ cells. Given is the average frequency per field with 60-fold magnification (_med ± SEM). * Gleason score.
<table>
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<tr>
<th>Tissue type</th>
<th>Characteristics</th>
<th>Age</th>
<th>Tumor stage</th>
<th>Tumor grade</th>
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<th>$^{%}$ IL-21$^+$ cells</th>
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<td>pT2, pN1a</td>
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<td>3+3=6$^*$</td>
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<td>23 ± 7</td>
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Figure 1

1A

CD19 PE-Cy7
anti-BCR
GrB APC

GrB B cells [%]

IL-2/IL-21 - IL-2 IL-21 - IL-2 IL-21

anti-BCR - - + + +

1B

* p < 0.03
** p < 0.005

IL-2/IL-21 - IL-2 IL-21 - IL-2 IL-21

anti-BCR - - + + +

1C

CD19

Side Scatter

CD4

IL-2/IL-21 - IL-2 IL-21 - IL-2 IL-21

Anti-BCR - - + + +

1D

T cell proliferation [%]

* p < 0.05

No Anti-BCR + Anti-BCR

1E

anti-BCR

Side Scatter

anti-BCR + IL-21

GrB substrate inhibitor

No GrB inhibitor Anti-GrB antibody

1F

T cell proliferation [%]

* p < 0.02
** p < 0.001

Anti-BCR Anti-BCR + IL-21

No GrB inhibitor Anti-GrB antibody GrB substrate inhibitor
Figure 2

** p < 0.002

** p < 0.005

TCR-ζ (17.9 kDa)

β-actin (42 kDa)

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<tr>
<td>Anti-GrB</td>
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Relative band intensity

TCR-ζ : β-actin

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</tr>
<tr>
<td>Anti-GrB antibody</td>
<td>-</td>
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Figure 3
Figure 4

**A**

Separate cultures of B cells were treated with medium, IL-2, or IL-21 alone or in combination with anti-BCR. The percentage of CD25+ cells was determined. The experiment was performed in triplicate. 

**B**

Coculture of B cells and T cells in a 1:1 ratio was performed with medium, IL-2, or IL-21 alone or in combination with anti-BCR. The percentage of CD25+ cells was determined. The experiment was performed in triplicate. 

**C**

The average IL-2 concentration [pg/ml] was measured in supernatants from separate and cocultures. The experiment was performed in triplicate. 

**D**

The IDO level was determined in supernatants from separate and cocultures. The experiment was performed in triplicate. 

**E**

The expression of IL-10 was determined in supernatants from separate and cocultures. The experiment was performed in triplicate. 

**F**

The expression of IL-2 was determined in supernatants from separate and cocultures. The experiment was performed in triplicate. 

**G**

The expression of CD25 was determined in supernatants from separate and cocultures. The experiment was performed in triplicate.
Figure 5
Breast carcinoma, highly differentiated, invasive and infiltrating growth

Breast carcinoma, intermediately differentiated, ductal invasive growth

Ovarian serous carcinoma, poorly differentiated

Ovarian mucinous adenocarcinoma, intermediately differentiated

Cervical squamous cell carcinoma, poorly differentiated, invasive growth

Cervical squamous cell carcinoma, intermediately differentiated, invasive growth

Ovarian carcinoma

Cervical carcinoma

Breast carcinoma
Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells

Stefanie Lindner, Karen Dahlke, Kai Sontheimer, et al.

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